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AUSTRALIA

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The University of Melbourne

PROVISIONAL SPECIFICATION

Invention Title:

Hydrogel Preparation and Process for Manufacture

The invention is described in the following statement:

Technical Field

The present invention relates to a separation medium, comprising a hydrogel preparation consisting of macropores and micropores produced by using a hydro-organic solvent.

5

Background Art

Hydrogels for separation processes

In many applications of separation processes, it is desirable to have a porous matrix with good water compatibility and mechanical properties. In general, two broad
10 classes of matrixes have been used. One general class is derived from water insoluble polymers by precipitation procedures such as Diffusion Induced Phase Separation (DIPS) and Thermally Induced Phase Separation (TIPS). These matrixes are relatively hydrophobic. A typical example is polysulphores membranes, which sometimes require surface treatment or modification by physical adsorption of hydrophilic polymers (e.g.
15 poly(vinyl alcohol)) to achieve satisfactory water wetting properties.

In many applications it is preferred to synthesize hydrogels from water-soluble monomers by incorporating crosslinking monomers into the polymer network. Typical examples are the range of hydrogels prepared by the free-radical co-polymerization of acrylamide and *N, N'*-methylenebisacrylamide. Such hydrogels are relative to DIPS
20 and TIPS more hydrophilic and more stable since the hydrophilic groups are an integral part of the polymer structure. It is well accepted that the range of monomers suitable for the production of such hydrogels is rather limited, and is restricted to the requirement that both the monomer and the corresponding polymer need to be soluble in the polymerization solvent.

To address this limitation, several attempts have been made to prepare hydrogels by the bulk polymerization of monomers that produce water insoluble polymers. It is well accepted that the porosity of such gels is dependent upon total monomer concentration of the reaction mixture. For example, hydrogels with higher total monomer content will have a tighter network structure because of increased inter-
30 penetration of polymer chains during network formation (Baker, J.; Hong, L.; Blanch, H.; Prausnitz, J. *Macromolecules* 1994, 27, 1446). As a result of this, and their high polymer content, hydrogels prepared in bulk are normally poor in mechanical strength (glassy and brittle), low in biocompatibility and water content, and possess a very limited pore size range. The absence of water in the synthesis environment of such hydrogels
35 also made subsequent solvent exchange with water difficult.

Polymerization-induced phase separation (PIPS) is a process in which an initially homogeneous solution of monomer and solvent becomes phase separated during the course of its polymerization. In hydrogel synthesis, PIPS can be induced by a number of factors: continuous increase in the fraction of molecules with high molecular weight, the unfavourable interactions between the polymer and other species in the reaction mixture, or the elasticity of the resultant polymeric network (Dušek, KJ. J. Polym. Sci. Polym. Symp. **1967**, 16, 1289; Boots, H.M.J.; Kloosterboer, J.G.; Serbútoviez, C.; Touwslager, F.J. *Macromolecules* **1996**, 29, 7683). Depending on the relative rates of the phase separation and the polymerization processes, PIPS can occur by the mechanism of nucleation-growth in the metastable region, or by spinodal decomposition in the multiphase coexisting region of the phase diagram (Eliçabe, G.E.; Larrondo, H.A.; Williams, R.J.J. *Macromolecules* **1997**, 30, 6550; Eliçabe, G.E.; Larrondo, H.A.; Williams, R.J.J. *Macromolecules* **1998**, 31, 8173).

In the homo-polymerizations of mono-vinyl monomer, during the course of the reaction, because of the continuous increase in the fraction of polymer in the reaction mixture, PIPS can occur if the polymers formed in the reaction mixture are not miscible with the polymerization solvent. For example, PIPS occurs at ~30% monomer conversion during the polymerization of a mixture composed of 30% 2-hydroxyethyl methacrylate and 70% water when the molecular weight of the resultant polymer is ~300,000; and at ~25% monomer conversion during the polymerization of a mixture composed of 20% acrylamide, 32.5% poly(ethylene glycol)-400 when the molecular weight of the resultant polymer is ~10,000.

Miscibility in a multi-component system is governed by its Gibbs free energy of mixing (ΔG_{mix}), which is a function of the enthalpies of mixing and the entropies of mixing between the various components in the mixture ($\Delta G_{\text{mix}} = \Delta H_{\text{mix}} - T\Delta S_{\text{mix}}$). Because the enthalpy of mixing between two chemically different polymers is mostly positive, increases in the average molecular weight of the polymer solution will decrease the overall entropy of the system. It is also expected to decrease the miscibility of the polymerization mixture. This leads to the occurrence of PIPS at lower monomer conversions. For example, the onset of PIPS is at ~1% monomer conversion during the polymerization of a mixture composed of 20% acrylamide, 32.5% poly(ethylene glycol)-400 when the molecular weight of the resultant polymer is ~5,500,000. Polymer systems with higher average molecular weight will be less miscible than corresponding systems with lower average molecular weight.

In a simplified gel formation process by the free radical co-polymerization of mono-vinyl monomer and multi-vinyl crosslinker, linear polymers are first formed in the solution during the fast propagation step, and later crosslinked with other molecules in close proximity by reaction through their pendent double bonds and additional monomer units (Stepho, R.F.T. "Non-linear polymerization, gelation and network formation, structure and properties", in Stepto, R.F.T. (ed.) Polymer Networks 1998; London, Blackie Academic & Professional, 14-63). Therefore, in a gel formation process, the average molecular weight of the polymer solution increases with increasing monomer conversion because of the ongoing crosslinking reactions.

Because hydrogels are defined as a network with infinite molecular weight which reaches the macroscopic dimensions of the sample itself (Flory P.J. Principles of polymer science. New York: Cornell University Press, 1953 (Chapter IX)), polymers with very high molecular weight are produced in the reaction mixture prior to the formation of a gel network. Such polymers are therefore expected to undergo phase separations when the polymerization solvent is immiscible with their corresponding linear polymer analogues with high molecular weight.

Acrylamide hydrogels, for separation in zone electrophoresis, were introduced in 1959 (Raymond, Weintraub, Science 1959, 130, 711) and widely used as matrices for gels, and other electrophoretic operations. For example, one membrane-based electrophoresis technique (Gradiflow™ (Gradipore, Australia)) involves a fixed boundary preparative electrophoresis method (US 5650055, US 5039386 and WO 0013776) and utilizes a thin acrylamide hydrogel membrane with a defined pore size (D.B. Rylatt, M. Napoli, D. Ogle, A. Gilbert, S. Lim, and C. H. Nair, J. Chromatog., A, 1999, 865, 145-153). However, despite its widespread popularity, there are several potential hazards and limitations which accompany the use of acrylamide hydrogel. For example, although the polymer is not toxic, exposure to the monomer and crosslinker during preparation of the gel poses significant health concerns. In addition, residual and derivative chemical present in the gel may also pose potential health concern.

Currently, the pore size range of commercially available membranes is somewhat limited, for example, large pores suitable for DNA and RNA separations are not routinely available. It is well known that for an acrylamide hydrogel, although an increase in pore size can be achieved by decreasing the polymer content, the mechanical strength and integrity will also be decreased. The loss of gel rigidity places a practical limit on the accessible size separation range of a given material. In order to overcome these problems and to obtain matrices of higher porosity, Righetti

(US 5785832) and Uriel (US 3578604) proposed polyacrylamide-agarose mixed-bed matrices. The matrix was obtained by a simultaneous but independent process of agarose and acrylamide gelification leading to an intertwining of the two polymers. The agarose used, however, is normally based on naturally occurring raw materials which often have associated chemical and structural impurities.

Righetti (US 5470916) described a process for synthesising polyacrylamide matrixes with large pores. It consists of adding, to the polymerization monomer mixture, hydrophilic polymers (e.g. polyethylene glycol, polyvinylpyrrolidone, hydroxymethyl cellulose) which, when added at a given concentration to the monomer mixture, force the chains to agglomerate together, thus forming a gel network having fibres of a much larger diameter than a regular acrylamide hydrogel. It was understood that the large pores were formed due to the competition between gelation and phase separation in the system (Asnaghi, D., Giglio, M., Bossi, A., Righetti, P.G., J. Mol. Strut. 1996, 38, 37). It is, however, hard to control the ranges of pore size obtainable using this technique.

Another approach to the synthesis of hydrogels with large pores is provided by template strategies (Beginn, U., Adv. Mater. 1998, 19, 16). This process resembles macroscopic metal casting processes in which templates preform the shapes of the pores like casting-cores are introduced into a liquid system and subsequently embedded by hardening of the solvent (i.e. polymerization). After removal of these cores from the surrounding matrix the shape of the voids that remain reflects the form of the templates.

Rill *et al.* (Rill, R.L., Locke, B.R., Liu, Y., Dharia, J., Van Winkle, D.L., Electrophoresis 1996, 17, 1304; Rill, R.L., Van Winkle, D.L., Locke, B.R., Anal. Chem. 1998, 70, 2433, Chakrapani, M., Van Winkle, D.H., Rill, R.L., Langmuir 2002, 18, 6449) reported templated acrylamide hydrogels as gel electrophoresis matrix and potential support for gel permeation chromatography. They showed that templating gels with sodium dodecyl sulfate (SDS) at concentrations up to 20% altered the electrophoretic separations of SDS-protein complexes in a manner consistent with the creation of pores by SDS micelles. Anderson (US 5244799) described a process in which templated hydrogels were created by polymerizing a mixture of a hydrophilic monomer, polymerizing agent, an ionic surfactant and water. However, the usage of surfactants as template also have a few limitations, such as i) foaming problems during the degassing and the polymerization process, ii) the need to equilibrate the monomer solution (Method from Anderson involve the equilibration of the monomer solution for at least a week), iii) in such procedures, it is difficult to completely remove the ionic surfactant from the hydrogel after the polymerization step. Anderson described an additional step in which the hydrogel was to be treated with a non-ionic surfactant solution while Rill *et al.*

reported the removal of 98% of SDS from the gel upon successive soaking in water. Residue ionic groups on the hydrogel matrix often caused undesirable electroosmotic properties when exposed to an electric field, and more importantly, were able to affect biomolecule separation by physical interactions with charged groups on them, and iv) high surfactant concentrations are required to form the necessary interconnecting templating pores. At such concentrations, polyacrylamide is often incompatible with the ionic surfactant, resulting in undesirable phase separation during the polymerization. For example, Antonietti *et al.* (Antonietti, M., Caruso, R.A., Goltner, C.G., Weissenberger, M.C. *Macromolecules* 1999, 32 1383) reported during the formation of a variety of polymer gels such as polyacrylamide in the presence of lyotropic surfactant mesophases that "prior to polymerization all mixtures are transparent, and become opaque or turbid-white shortly after the start of the reaction". Rill also reported that gels formed in the presence of 30% or more SDS became uniformly white as the surfactants were removed.

Undesirable swelling or shrinking has always been a drawback in the use of acrylamide hydrogels in non-aqueous operating systems such as the separation of ions in non-aqueous systems and the electrophoretic separation of hydrophobic proteins using organic solvents. Hydrogels synthesised in a solvent similar to that of its final operating environment will be more tolerant to solvent compositional changes. Typical solvents used in non-aqueous operating systems include alcohols, glycols, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), tetramethylurea, formamide, tetramethylene sulfone, chloral hydrate N-methyl acetamide, N-methyl pyrrolidone and phenol. It is, however, well known that when amounts of water miscible solvents such as DMF, DMSO, TMU, ethylene glycol, or propylene glycol are added to the acrylamide polymerization mixture, the mechanical strength and clearness of the polymer gel are severely compromised.

Amphiphilic polymer networks of α,ω -(meth)acryloyloxy monomers such as poly(2-hydroxyethyl methacrylate) (poly(HEMA)) have been studied extensively as materials for pharmaceutical and biomedical applications, including carriers for controlled drug delivery and materials for prosthetic devices. The mechanical strength provided by the hydrophobic backbone and the hydrophilicity of the hydroxy and ester groups on the polymer side chains make polymers produced from HEMA excellent candidates for hydrogels for separation processes. Zewert and Harrington (US 5290411; US 5290411; Zewert, T., Harrington, M., *Electrophoresis* 1992, 13, 817-824; Zewert, T., Harrington, M., *Electrophoresis* 1992, 13, 824), and Solomon et al.

(PCT AU01/01632) have described the usage of hydrogels prepared from α,ω -(meth)acryloyloxy monomers in various electrophoretic operations.

Most existing 2-Hydroxyethyl methacrylate (HEMA) systems are prepared in bulk, or with < 50% diluent. Owing to the hydrophobicity of the network, organic diluents such as ethylene glycol and di(ethylene glycol) are normally used (WO 0044356; Caliceti, P., Veronese, F., Schiavon, O., *Il Farmaco* **1992**, 47, 275, Carenza, M., *Radiat. Phys. Chem.* **1993**, 42, 897). Although the properties of these hydrogels can be modified by crosslinking or by the use of different diluents, their swelling in water is thermodynamically limited to ~40% (Havsky, M., Prins, W., *Macromolecules* **1970**, 3, 415; Nakamura, K., Nakagawa, T., *Journal of Polymer Science* **1975**, 13, 2299).

As a result, such HEMA hydrogels are normally poor in mechanical strength (glassy and brittle), low in biocompatibility, low in-water content, and possess a very limited pore size range. The absence of water in the synthesis environment of such hydrogels also made subsequent solvent exchange with water difficult. In addition, the toxicity of some of the diluents is of great concern. Such hydrogels have been predominantly used in applications that desire low water swelling, such as contact lenses and transport membranes for gases and ions (Corkhill, P.H., Jolly, A.M., Ng, C.O., Tighe, B.J. *Polymer* **1987**, 28, 1758; Hamilton, C.J., Murphy, S.M., Atherton, N.D., Tighe, B.J., *Polymer* **1988**, 29, 1879).

It is well accepted that the porosity of such hydrogels is dependent upon the particular monomer, particular crosslinking agent, and the degree of crosslinking. For example, hydrogels with higher total monomer content will have a tighter network structure because of increased interpenetration of polymer chains during network formation (Baker, J.; Hong, L.; Blanch, H.; Prausnitz, J. *Macromolecules* **1994**, 27, 1446). It is thus highly desirable to be able to produce an HEMA hydrogel with high water content at a low initial concentration of monomers (<50 wt%) in order to obtain the desired biocompatibility and pore sizes for applications such as electrophoresis separation membranes.

Several attempts have been made to improve the water swelling properties of HEMA hydrogels and to prepare such gel at a low initial concentration of monomers.

i) HEMA hydrogels were synthesised in various hydro-organic solvents. Refojo (Refojo, M., *Journal of Polymer Science: Part A-1* (1967), 5, 3103) reported that visually clear hydrogels of poly(2-hydroxyethyl methacrylate) may be prepared by conducting the polymerization in ethylene glycol-water solution. The phase separation limit for this type of system was reported to be 45% of water in the reaction solution, allowing the total

monomer concentrations to be decreased by the replacement of monomers with diluent (Warren, T., Prins, W., *Macromolecules* (1972), 5, 506). In addition to the fact that HEMA hydrogels prepared in such diluent were reported to exhibit a narrow range of swelling at equilibrium in water (41% water) regardless of the initial dilution of the monomer solution and relatively low level of crosslinking. Results from our laboratory have shown that this separation limit is highly dependent upon both the amount of crosslinker and the choices of diluent in the reaction solution, with some formulations forming heterogeneous opaque polymer mass even when the water content is below 45%. Zewert and Harrington (Zewert, T., Harrington, M., *Electrophoresis* 1992, 13, 817) reported HEMA hydrogel synthesis in aqueous sulfolane solution and concluded that HEMA polymerization is thoroughly incompatible with sulfolane even if sulfolane concentrations are as low as 10%.

ii) Various HEMA derivatives such as the poly(alkylene glycol) esters of acrylic or methacrylic acid (e.g. poly(ethylene glycol) methacrylate) were used instead of HEMA to prepare hydrogels with improved water swelling properties. The disadvantages of such monomers is that they are expensive and difficult to prepare. In addition, the pore size of hydrogels prepared by these monomers are also limited because of their large molecular weight, restricting the number of monomer units available in the monomer mixture.

iii) In order to obtain HEMA hydrogels with improved water swelling properties, it is common to copolymerize HEMA with a hydrophilic monomer such as acrylamide. Bajpai and Shrivastava (Bajpai, A.K., Shrivastava, M. J. *Biomater. Sci. Polymer Edn* 2002, 13, 237) copolymerised HEMA with acrylamide (% acrylamide > 40 mol %) in the presence of a hydrophilic polymer, poly(ethylene glycol) (PEG, MW 600). It was found that the swelling ratio of such hydrogel increases with increasing PEG 600 content in the monomer mixture to a maximum at 4.31% (by weight). Such hydrogels, according to the authors, "could be regarded as a network of poly(ethylene glycol) and poly(HEMA-co-acrylamide) chains thus creating free volumes of varying meshes for accommodating penetration of water molecules". It was also stated by Bajpai and Shrivastava that there is no clear advantage of using a highly hydrophilic polymer content - "beyond 0.56 of PEG (600) content (4.31%), the network density of the gel may become so high that mesh sizes of free volumes available between the network chains get reduced... thus decreasing the swelling of the gel". It is clear that the co-polymerization of acrylamide with HEMA does not eliminate the disadvantages associated with acrylamide hydrogels.

The present inventors have now developed new hydrogels suitable for a number of separation techniques. The present invention also provides visually clear hydrogels with good water compatibility and swelling properties to be synthesized from monomers in hydro-organic or organic solvents.

5

Disclosure of Invention

In a first aspect, the present invention provides a process for producing a polymeric hydrogel having a network containing macropores and micropores, the process comprising:

- 10 (a) forming a mixture by adding of at least one monomer having at least one double bond, at least one crosslinker having at least two double bonds, an initiation system and a polymeric additive to form a hydro-organic system with water; and
- (b) allowing the monomer and crosslinker to copolymerize to form a hydrogel having a polymeric network containing macropores and micropores.

- 15 The monomer having at least one double bond may be selected from esters of acrylic and methacrylic acid, or polyol esters of acrylic or methacrylic acid. Typical polyols are polyethylene glycol, a range of polyethylene glycol esters or ethers, polypropylene glycol, a range of polypropylene glycol esters or ethers, random or block copolymers of ethylene glycol and propylene glycol, or any suitable polyols such as
- 20 glycerol, pentaerythritol, ethylene glycol or propylene glycol which are fully or partly esterified. Mixtures consist of at least two of the above monomers can also be used.

Mixtures of the above monomer with any other well-known monomers suitable for free radical polymerization may be used.

- 25 Preferably use of above monomer with greater than 50% in the mixture of monomers; more preferably greater than 80%.

Preferably, the monomer is one or more hydrophilic monomers from the esters of acrylic acids.

In one preferred form, the monomer is hydroxyethyl methacrylate (HEMA).

- 30 The crosslinker having at least two double bond may be selected from esters of acrylic and methacrylic acid, or polyol esters of acrylic or methacrylic acid. Typical polyols are polyethylene glycol, a range of polyethylene glycol esters or ethers, polypropylene glycol, a range of polypropylene glycol esters or ethers, random or block copolymers of ethylene glycol and propylene glycol, or any suitable polyols such as glycerol, pentaerythritol, ethylene glycol or propylene glycol which are fully or partly

esterified (for example, glycerol can be esterified with methacrylic acid to give the crosslinking mixture). Mixtures consist of at least two of the above crosslinkers can also be used.

5 Mixtures of above crosslinker with any other well-known crosslinkers suitable for free radical polymerization may be used.

Preferably use of the above crosslinker with greater than 50% in the mixture of crosslinkers; more preferably greater than 80%.

In one preferred form, the crosslinker is ethylene glycol dimethacrylate (EGDMA).

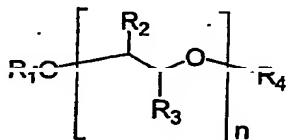
10 Preferably, the polymeric hydrogel is made from a mixture of monomer content of about 10 to 40%M and crosslinker of about 1 to 30%X before polymerization. When HEMA and EGDMA are used, the preferred compositions of monomer mixture of HEMA with EGDMA are less than about 40% M and less than about 20% X. It will be appreciated, however, that other concentrations can be used depending on the
15 monomer and crosslinker used.

Any suitable free radical producing method can be used as the initiation system. The initiation system is preferably formed by the redox, thermal or photo initiator/s. More preferably, the redox initiator is formed by ammonium persulphate (APS) with N,N,N',N'-tetramethylethylenediamine (TEMED).

20 The polymeric additive is preferably a hydrophilic polymer miscible with water and miscible with a linear polymer produced from the monomer used for copolymerization; or a hydrophilic polymer miscible with water and has a similar solubility parameter ($\pm 10(\text{MPa})^{0.5}$) to that of a polymer produced from the monomer used for copolymerization. The polymeric additive can be single entity acting as both a
25 porogen to form macropores during the polymerization and a solvent with water to form the hydro-organic solvent.

The polymeric additive is preferably selected from ethylene glycol or polyethylene glycol, propylene glycol or polypropylene glycol, random or block copolymers of any of the above mixtures, or any of the above additives that have an
30 ester or ether end group. Mixtures consist of at least two of the additives can also be used.

More preferably, the polymeric additive has the following general formulation:



$\text{R}_1, \text{R}_4 = \text{H}, \text{CH}_3, -(\text{CH}_2)_x\text{CH}_3 (x=1-4),$
 $-\text{C}(=\text{O})-\text{R}_5 (\text{R}_5=(\text{CH}_2)_x\text{CH}_3 (x=0-4))$

$\text{R}_2, \text{R}_3 = \text{H}, \text{CH}_3, -(\text{CH}_2)_x\text{CH}_3 (x=1-4), \text{OH}$

In a preferred form, the polymeric additive is a polyethylene glycol or polypropylene glycol. The polyethylene glycol preferably has a molecular weight range from about 100 to 100000; preferably from about 200 to 10000; more preferably from about 400 to 4000.

The polypropylene glycol typically has a molecular weight range from about 100 to 100000; preferably from 200 to 10000; more preferably from about 58 to 600.

In another preferred form, the polymeric additive is a copolymer with a hydrophilic component and a hydrophobic component. Preferably, the polymeric additive is a copolymer of polyethylene glycol with polypropylene glycol.

In use, the polymeric hydrogel formed can be used in the hydro-organic solvent or the hydro-organic solvent components exchanged with water.

In a second aspect, the present invention provides a polymeric hydrogel having a network containing macropores and micropores produced by the method according to the first aspect of the present invention.

In a third aspect, the present invention provides a polymeric hydrogel comprising a network of macropores and micropores formed by copolymerizing at least one monomer having at least one double bond and at least one crosslinker having at least two double bonds in the presence of a polymeric additive forming a hydro-organic system with water.

The monomer having at least one double bond may be selected from esters of acrylic and methacrylic acid, or polyol esters of acrylic or methacrylic acid. Typical polyols are polyethylene glycol, a range of polyethylene glycol esters or ethers, polypropylene glycol, a range of polypropylene glycol esters or ethers, random or block copolymers of ethylene glycol and propylene glycol, or any suitable polyols such as glycerol, pentaerythritol, ethylene glycol or propylene glycol which are fully or partly esterified. Mixtures consist of at least two of the above monomers can also be used.

Mixtures of the above monomer with any other well-known monomers suitable for free radical polymerization may be used.

Preferably use of above monomer with greater than 50% in the mixture of monomers; more preferably greater than 80%.

- 5 Preferably, the monomer is one or more hydrophilic monomers from the esters of acrylic acids.

In one preferred form, the monomer is hydroxyethyl methacrylate (HEMA).

- 10 The crosslinker having at least two double bond may be selected from esters of acrylic and methacrylic acid, or polyol esters of acrylic or methacrylic acid. Typical polyols are polyethylene glycol, a range of polyethylene glycol esters or ethers, polypropylene glycol, a range of polypropylene glycol esters or ethers, random or block copolymers of ethylene glycol and propylene glycol, or any suitable polyols such as glycerol, pentaerythritol, ethylene glycol or propylene glycol which are fully or partly esterified (for example, glycerol can be esterified with methacrylic acid to give the crosslinking mixture). Mixtures consist of at least two of the above crosslinkers can also be used.

Mixtures of above crosslinker with any other well-known crosslinkers suitable for free radical polymerization may be used.

- 20 Preferably use of the above crosslinker with greater than 50% in the mixture of crosslinkers; more preferably greater than 80%.

In one preferred form, the crosslinker is ethylene glycol dimethacrylate (EGDMA).

- 25 Preferably, the polymeric hydrogel is made from a mixture of monomer content of about 10 to 40%M and crosslinker of about 1 to 30%X before polymerization. When HEMA and EGDMA are used, the preferred compositions of monomer mixture of HEMA with EGDMA are less than about 40% M and less than about 20% X. It will be appreciated, however, that other concentrations can be used depending on the monomer and crosslinker used.

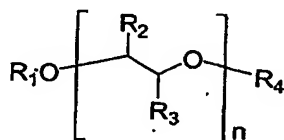
- 30 Any suitable free radical producing method can be used as the initiation system. The initiation system is preferably formed by the redox, thermal or photo initiator/s. More preferably, the redox initiator is formed by ammonium persulphate (APS) with *N,N,N',N'*-tetramethylethylenediamine (TEMED).

The polymeric additive is preferably a hydrophilic polymer miscible with water and miscible with a linear polymer produced from the monomer used for

copolymerization; or a hydrophilic polymer miscible with water and has a similar solubility parameter ($\pm 10(\text{MPa})^{0.5}$) to that of a polymer produced from the monomer used for copolymerization. The polymeric additive can be single entity acting as both a porogen to form macropores during the polymerization and a solvent with water to form the hydro-organic solvent.

The polymeric additive is preferably selected from ethylene glycol or polyethylene glycol, propylene glycol or polypropylene glycol, random or block copolymers of any of the above mixtures, or any of the above additives that have an ester or ether end group. Mixtures consist of at least two of the additives can also be used.

More preferably, the polymeric additive has the following general formulation:



$\text{R}_1, \text{R}_4 = \text{H}, \text{CH}_3, -(\text{CH}_2)_x-\text{CH}_3 \text{ (} x=1-4 \text{)},$
 $-\text{C}(=\text{O})-\text{R}_5 \text{ (} \text{R}_5=(\text{CH}_2)_x-\text{CH}_3 \text{ (} x=0-4 \text{))}$

$\text{R}_2, \text{R}_3 = \text{H}, \text{CH}_3, -(\text{CH}_2)_x-\text{CH}_3 \text{ (} x=1-4 \text{)}, \text{OH}$

In a preferred form, the polymeric additive is a polyethylene glycol or polypropylene glycol. The polyethylene glycol preferably has a molecular weight range from about 100 to 100000; preferably from about 200 to 10000; more preferably from about 400 to 4000.

The polypropylene glycol typically has a molecular weight range from about 100 to 100000; preferably from 200 to 10000; more preferably from about 58 to 600.

In another preferred form, the polymeric additive is a copolymer with a hydrophilic component and a hydrophobic component. Preferably, the polymeric additive is a copolymer of polyethylene glycol with polypropylene glycol.

Preferably, the mixture is degassed to remove any dissolved oxygen prior to polymerization.

In use, the polymeric hydrogel formed can be used in the hydro-organic solvent or the hydro-organic solvent components exchanged with water.

In a fourth aspect, the present invention provides a separation medium formed from the polymeric hydrogel according to the second or thirds aspects of the present invention.

5 Preferably, the separation medium is in the form of membrane, slab, beads or column. The medium is particularly suitable as an electrophoretic medium capable of separating large biomolecules having a molecular weight of at least 2000 k.

In a fifth aspect, the present invention provides a visually clear polymeric hydrogel produced according to the second or thirds aspects of the present invention.

10 The present inventors have found that by the use of mixtures of water and water-miscible entities as the polymerization solvent, visually clear hydrogels can be prepared even when the polymerization solvent is immiscible with the corresponding linear polymer analogues. For example, a mixture of 20% poly(acrylamide)-5,500,000, 1% poly(vinyl alcohol)-18,000 (88% hydrolyzed), and 79% water is immiscible, but the polymerization of 20% solutions of acrylamide and *N,N'*-methylenebisacrylamide can
15 give visually clear gels; a mixture of 15% poly(2-hydroxyethyl methacrylate)-300,000, 75% ethylene glycol dimethyl ether or 75% poly(ethylene glycol) dimethyl ether, and 10% water is immiscible, but the polymerization of 15% solutions of 2-hydroxyethyl methacrylate and ethylene glycol dimethacrylate in these solvents can give visually clear gels.

20 These results are new and unexpected because the general teaching from most scientific literature on monomer selection for hydrogel synthesis is that the polymerization solvent should be a solvent for the linear analogues of the resultant polymeric network.

25 By the selection of the water-miscible entities, the 'freezing point' of the reaction mixture can be controlled such that it occurs at a monomer conversion lower than the critical monomer conversion for the onset of PIPS. The 'freezing point' of the reaction mixture is defined as the critical monomer conversion at which the viscosity of the mixture reaches a specific level when the mobility of polymer chains in the mixture becomes negligible and the dynamic concentration fluctuations of pre-gel polymer
30 solutions are frozen in the final network structure. The resultant hydrogels of these systems will be visually clear and have a relatively uniform network because the polymer mixture was frozen in its miscible state before phase separation could occur. Hydrogels prepared by this approach have superior swelling, optical, and mechanical properties to that prepared by systems that reaches the phase boundary before the gel point. Those
35 gels are formed from dispersions of precipitated polymers in the liquid phase (Okay O.

Polymer 1999, 40, 4117) and are highly opaque polymer masses that have very different properties from hydrogels synthesized using our approach.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to
5 imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a
10 context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

In order that the present invention may be more clearly understood, preferred
15 forms will be described with reference to the following drawings and examples.

Brief Description of the Drawings

Figure 1 shows migration ratios of Kaleidoscope Pre-stained Standards in 10%M
2%X acrylamide gel cassette synthesized in water, aqueous solutions of ethylene glycol
20 (25%) or propylene glycol (25%).

Figure 2 shows migration ratios of SDS-PAGE Molecular Weight Standards (board range) in 10%M 2%X acrylamide gel cassette synthesized in water, or aqueous solutions of poly(ethylene glycol).

Figure 3 shows migration ratios of SDS-PAGE Molecular Weight Standards
25 (board range) in 10%M 2%X acrylamide gel cassette synthesized in water or aqueous solutions of tri(ethylene glycol) and poly(ethylene glycol).

Figure 4 shows migration ratios of Kaleidoscope Prestained Standards in 10%M
2%X acrylamide gel cassette synthesized in water and aqueous solutions of tri(ethylene glycol).

30 Figure 5 shows turbidity results of polymers synthesized according to Example 29.

Figure 6 shows turbidity results of polymers synthesized according to Example 30.

Figure 7 shows turbidity results of polymers synthesized according to Example 31.

Figure 8 shows turbidity results of polymers synthesized according to Example 32.

5 Figure 9 shows turbidity results of polymers synthesized according to Example 33.

Figure 10 shows turbidity results of polymers synthesized according to Example 34.

10 Figure 11 shows turbidity results of polymers synthesized according to Example 35.

Figure 12 shows turbidity results of polymers synthesized according to Example 36.

Figure 13 shows turbidity results of polymers synthesized according to example 37.

15 Figure 14 shows turbidity results of polymers synthesized according to Example 38.

Figure 15 shows the separation and migration pattern of Bovine serum albumin (MW 67,000) by a 15%M 4%X HEMA/EGDMA membrane synthesized in 80% aqueous PEG 200 solution (Example 41) using 40 mM MES bis-TRIS buffer.

20 Figure 16 shows turbidity results of polymers synthesized according to Example 56.

Figure 17 shows a schematic diagram of the formation process of 20%M acrylamide hydrogels in the presence of water and a water-soluble entity. Line E represents systems with 0%X; line F, 2%X; line G, 3%X; line H, 10%X.

25 Figure 18 shows real-time viscosity measurements of the polymerization of 20%M acrylamide solutions, in the presence of 17.5% PEG-400, at various %X. Time at which phase separation was observed in the samples are represented by dark coloured points (circle).

Mode(s) for Carrying Out the Invention

Newel formulations for HEMA hydrogel synthesis

The present inventors have developed a new synthesis method using a mixture of water and water-miscible entities as the polymerization solvent such that HEMA hydrogels can be crosslinked with ethylene glycol dimethacryate (EGDMA) using low initial monomer content (5-50%). Using water-miscible entities such as polymers with repeating ethoxylated and propyoxylated units (e.g. poly(ethylene glycol) and poly(propylene glycol) or random or block copolymers of poly(ethylene glycol) at a polymeric-additive glycol-water ratio of about 9:1 to 1:9), hydrogels based on HEMA were successfully formed having higher water swelling properties and bigger pore sizes than those produced previously. Such hydrogels can be subsequently used as synthesized or after the water-miscible entities have been displaced with water. This result is unexpected, given that it is well known that high concentrations of hydrophilic polymer (i.e. poly(ethylene glycol) and poly(propylene glycol)) in acrylamide hydrogel synthesis would lead to phase separation of the reaction mixture. For example, Righetti (Righetti, P.G Chromatogr. A 1995, 698, 3) observed that when acrylamide hydrogels were synthesised in the presence of PEG 2000-20,000, turbid gels (phase separation) were produced and was a function of both length and concentration of the polymer. It was observed that longer polymer chains induce phase separation at lower concentration; all gels become turbid when the PEG concentration in the solvent exceed 10 wt%.

It was also discovered by the present inventors that as the molecular weight of the water-miscible entities increases, the pore size of the hydrogels becomes dependent upon the properties of the entities, with the entities acting as a "template". In high molecular weight solvents, hydrogels synthesized in solutions of high molecular weight entities were observed to swell more than that of lower molecular weight. To our knowledge, this is the first system in which the templating system is also acting as the solvent for the hydrogel.

Multimodal hydrogels

Utilising the templating and the solvent properties of the water-miscible entities, it was discovered that multimodal HEMA hydrogels can be obtained by careful selection of the concentrations of monomer, the crosslinking extent, and the types and concentrations of water-miscible entities in a one-step process. Two general types of

pores exist in such membranes - macropores formed by the template, and micropores formed by the crosslinking of polymer chains. Dependent upon the concentrations of the water-miscible entities, the macropores in the hydrogel can be continuous (i.e. interconnected), or non-continuous.

5 Derivatives of monomers such as the poly(alkylene glycol) esters of acrylic or methacrylic acid can also be used in the same manner as HEMA to prepare hydrogels with multimodal channels.

Such hydrogels are different from these synthesised by Zewert and Harrington (US 5290411 and US 5290411) because:

10 i) Their teaching indicates that the pore size of the gel is dependent upon the types and concentration of monomer and crosslinkers. Pore sizes of hydrogels according to the present invention are not only dependent upon the types and concentration of monomer and crosslinkers but also dependent upon the size of the water-miscible entities;

15 ii) The present hydrogels have two types of pores within its network, macropores and micropores;

20 iii) In the patent of Zewert and Harrington, organic solvents were added mainly for the usage of the resultant gel in organic electrophoresis and were not subsequently replaced with water. In the present invention, the water-miscible entities are acting both as a solvent and a template, and are subsequently exchanged with water.

Applications

HEMA hydrogels made with the above formulations are particularly well-suited for use as separation membranes for biomolecules. Other related areas of interest
25 include biocompatible applications such as prosthetic devices, drug releases matrixes, and tissue scaffolds.

Membrane-Based Electrophoresis

30 A number of membrane-based electrophoresis apparatus developed by Gradipore Limited, Australia were used in the following experiments. In summary, the apparatus typically included a cartridge which housed a number of membranes forming two chambers, cathode and anode connected to a suitable power supply, reservoirs for samples, buffers and electrolytes, pumps for passing samples, buffers and electrolytes,

and cooling means to maintain samples, buffers and electrolytes at a required temperature during electrophoresis.

The cartridge contained three substantially planar membranes positioned and spaced relative to each other to form two chambers through which sample or solvent can be passed. A separation membrane was positioned between two outer membranes (termed restriction membranes as their molecular mass cut-offs are usually smaller than the cut off of the separation membrane). When the cartridge was installed in the apparatus, the restriction membranes were located adjacent to an electrode. The cartridge is described in AU 738361, which description is incorporated herein by reference.

Description of membrane-based electrophoresis can be found in US 5039386 and US 5650055 in the name of Gradipore Limited, which description is incorporated herein by reference.

15 **Polyacrylamide Gel Electrophoresis (PAGE)**

Standard PAGE methods were employed as set out below.

Reagents: 10x SDS Glycine running buffer (Gradipore Limited, Australia), dilute using Milli-Q water to 1x for use; 1x SDS Glycine running buffer (29 g Trizma base, 144 g Glycine, 10 g SDS, make up in RO water to 1.0 L); 10x TBE II running buffer (Gradipore), dilute using Milli-Q water to 1x for use; 1x TBE II running buffer (10.8 g Trizma base, 5.5 g Boric acid, 0.75 g EDTA, make up in RO water to 1.0 L); 2x SDS sample buffer (4.0 ml, 10% (w/v) SDS electrophoresis grade, 2.0 ml Glycerol, 1.0 ml 0.1% (w/v) Bromophenol blue, 2.5 ml 0.5M Tris-HCl, pH 6.8, make up in RO water up to 10 ml); 2x Native sample buffer (10% (v/v) 10x TBE II, 20% (v/v) PEG 200, 0.1g/L Xylene cyanole, 0.1g/L Bromophenol blue, make up in RO water to 100%); Coomassie blue stain (Gradipure™, Gradipore Limited). Note: contains methanol 6% Acetic Acid solution for de-stain.

Molecular weight markers (Recommended to store at -20°C): SDS PAGE (e.g. Sigma wide range); Western Blotting (e.g. color/ rainbow markers).

30 **SDS PAGE with non-reduced samples**

To prepare the samples for running, 2x SDS sample buffer was added to sample at a 1: 1 ratio (usually 50 μ L / 50 μ L) in the microtiter plate wells or 1.5 ml tubes. The samples were incubated for 5 minutes at approximately 100°C. Gel cassettes were clipped onto the gel support with wells facing in, and placed in the tank. If only running one gel on a support, a blank cassette or plastic plate was clipped onto the other side of the support

Sufficient 1x SDS glycine running buffer was poured into the inner tank of the gel support to cover the sample wells. The outer tank was filled to a level approximately midway up the gel cassette. Using a transfer pipette, the sample wells were rinsed with the running buffer to remove air bubbles and to displace any storage buffer and residual polyacrylamide.

Wells were loaded with a minimum of 5 μ l of marker and the prepared samples (maximum of 40 μ l). After placing the lid on the tank and connecting leads to the power supply the gel was run at 150V for 90 minutes. The gels were removed from the tank as soon as possible after the completion of running, before staining or using for another procedure (e.g. Western blot).

Staining and De-staining of Gels

The gel cassette was opened to remove the gel which was placed into a container or sealable plastic bag. The gel was thoroughly rinsed with tap water, and drained from the container. Coomassie blue stain (approximately 100 ml Gradipure™, Gradipore Limited, Australia)) was added and the container or bag sealed. Major bands were visible in 10 minutes but for maximum intensity, stained overnight. To de-stain the gel, the stain was drained off from the container.

The container and gel were rinsed with tap water to remove residual stain. 6% acetic acid (approximately 100 ml) was poured into the container and sealed. The de-stain was left for as long as it takes to achieve the desired level of de-staining (usually 12 hours). Once at the desired level, the acetic acid was drained and the gel rinsed with tap water.

DEFINITIONS

The following terms shall have the indicated definitions unless otherwise indicated:

"Hydrogel" is a chemically crosslinked polymer characterized by hydrophilicity and insolubility in water.

"Micropores" are pores within the gel network of the background matrix. The size of these pores can be related to the hydrogel formation species in the initial pre-gelling mixture using relationships and theories developed for common electrophoretic matrixes. For example, micropores within an acrylamide hydrogel are related to the total monomer concentration and monomer to crosslinker ratios in the free radical polymerization of acrylamide and N,N'-methylenebisacrylamide (Bansil, R.; Gupta, M. *Ferroelectrics* 1980, 30, 64).

"Macropores" are pores within the membrane that are significantly larger (more than 2 times) than micropores of the background matrix.

"Microporous membrane" is a separation membrane having substantially continuous interconnecting micropores. Such membranes are used extensively in preparative electrophoresis.

"Macroporous membrane" is a separation membrane having continuous interconnecting micropores but non-continuous macropores (i.e. macropores are not connected directly to each other). Such membranes have similar sieving properties to the corresponding microporous membrane, but allows for higher flow rate through the matrix because of the reduced diffusional constraints.

The term "stream 1 (S1)" refers to denote the first interstitial volume where sample is supplied in a stream to the electrophoresis apparatus. This stream may also be called the "upstream".

The term "stream 2 (S2)" is used in this specification to denote the second interstitial volume where material is moved from the first interstitial volume through the separation membrane to a stream of the electrophoresis apparatus. This stream may also be called the "downstream".

The term "forward polarity" is used when the first electrode is the cathode and the second electrode is the anode in the electrophoresis apparatus and current is applied accordingly.

The term "reverse polarity" is used when polarity of the electrodes is reversed such that the first electrode becomes the anode and the second electrode becomes the cathode.

25 ABBREVIATIONS

Acrylamide (AAM), N,N'-methylenebisacrylamide (BIS), poly(acrylamide) gel electrophoresis (PAGE), 2-hydroxyethyl acrylate (HEA), 2-hydroxyethyl methacrylate (HEMA), poly(ethylene glycol) acrylate (PEGA), poly(ethylene glycol) methacrylate (PEGMA), ethylene glycol diacrylate (EGDA), ethylene glycol dimethacrylate (EGDMA), poly(ethylene glycol) acrylate (PEGA), poly(ethylene glycol) methacrylate (PEGMA), poly(ethylene glycol) diacrylate (PEGDA), poly(ethylene glycol) dimethacrylate (PEGDMA), poly(ethylene glycol) PEG, and poly(propylene glycol) PPG; poly(ethylene glycol) methyl ether PEGME; N,N,N',N'-tetramethylethylenediamine (TEMED); ammonium persulfate (APS).

EXAMPLES

Example 1: Preparation of monomer solutions

Two terms are introduced to classify the monomer solutions:

%M refers to the total concentration of monomer as a weight percentage;

5 %X refers to the number of double bonds on the crosslinkers as a portion of the total number of double bonds on the monomers.

$$\%M = \frac{\text{total mass of monomers (g)}}{\text{mass of reaction mixture (g)}} \times 100$$

$$\%X = \frac{\text{number of double bonds on crosslinkers (mol)}}{\text{total number of double bonds on monomers (mol)}} \times 100$$

10 *Preparation of acrylamide hydrogels*

Example 2: Preparation of 10%M 2%X AAm/BIS hydrogels for swelling tests using water as solvent

15 Monomer solutions (10 g) were prepared by dissolving AAm (978.3 mg) and BIS (21.7 mg) in water (9 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment.

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Example 3: Preparation of 10%M 2%X AAm/BIS hydrogels for swelling tests using aqueous ethylene glycol as solvent

25 Aqueous solutions of ethylene glycol (25, 50 and 75%) were prepared by varying amounts of ethylene glycol and water. AAm (978.3 mg) and BIS (21.7 mg) were added to the above solutions (9 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 4: Preparation of 10%M 2%X AAm/BIS hydrogels for swelling tests using aqueous propylene glycol as solvent

Aqueous solutions of propylene glycol (25, 50 and 75%) were prepared by varying amounts of ethylene glycol and water. AAm (978.3 mg) and BIS (21.7 mg) were added to the above solutions (9 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 5: Preparation of 10%M 2%X AAm/BIS hydrogels for swelling tests using aqueous tri(ethylene glycol) as solvent

Aqueous solutions of triethylene glycol (22, 44, 67 and 72%) were prepared by varying amounts of triethylene glycol and water. AAm (978.3 mg) and BIS (21.7 mg) were added to the above solutions (9 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 6: Preparation of 10%M 2%X AAm/BIS hydrogels for swelling tests using aqueous poly(ethylene glycol) 400 as solvent

Aqueous solutions of poly(ethylene glycol) 400 (6, 11, 16 and 22%) were prepared by varying amounts of poly(ethylene glycol) 400 and water. AAm (978.3 mg) and BIS (21.7 mg) were added to the above solutions (9 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 7: Preparation of 10%M 2%X AAm/BIS hydrogels for turbidity measurements using aqueous tri(ethylene glycol) as solvent

Aqueous solutions of tri(ethylene glycol) (11, 22, 33, 44, 55, 61, 64, 66, 69 and 72%) were prepared by varying amounts of tri(ethylene glycol) and water. AAm (978.3 mg) and BIS (21.7 mg) was added to the above solutions (9 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 8: Preparation of 10%M 2%X AAm/BIS hydrogels for turbidity measurements using aqueous poly(ethylene glycol) 400 as solvent

Aqueous solutions of poly(ethylene glycol) 400 (6, 11, 16, 19, 22, 27 and 33%) were prepared by varying amounts of poly(ethylene glycol) 400 and water. AAm (978.3 mg) and BIS (21.7 mg) were added to the above solutions (9 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 9: Preparation of 10%M 2%X AAm/BIS hydrogels for turbidity measurements using aqueous poly(ethylene glycol) 400 as solvent at 40°C

Aqueous solutions of poly(ethylene glycol) 400 (6, 11, 16, 19, 22, 27 and 33%) were prepared by varying amounts of poly(ethylene glycol) 400 and water. AAm (978.3 mg) and BIS (21.7 mg) were added to the above solutions (9 g) in disposable glass vials. The monomer solution was then placed in a 40°C water bath for 15 mins and degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the polymerization was then allowed to proceed at 40°C for 2 hr under an argon environment.

Example 10: Preparation of 10%M 2%X AAm/BIS hydrogels for turbidity measurements using aqueous poly(ethylene glycol) 20,000 as solvent

Aqueous solutions of poly(ethylene glycol) 20,000 (0.02, 0.04, 0.06, 0.08, 0.1, 0.12 and 0.14%) were prepared by varying amounts of poly(ethylene glycol) 20,000 and water. AAm (978.3 mg) and BIS (21.7 mg) were added to the above solutions (9 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 11: Preparation of 10%M 2%X AAm/BIS hydrogel cassettes for gel electrophoresis using water as solvent

10%M 2%X solutions (10 g) were prepared by dissolving AAm (978.3 mg) and BIS (21.7 mg) in water (6.5 g) and 1.5M Tris-HCl buffer (pH 8.8, 2.5 g). The stock buffer solution was prepared by dissolving Tris (27.23 g) in water (80 ml) and adjusted to the pH of 8.8 with 6 N HCl followed by making up the required volume (150 ml) with water.

The monomer solution was degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS (64.1 μ m) and 10% (v/v) TEMED (42.4 μ m). The gel solution (7 ml) was then immediately cast between two glass plates (8 x 8 cm, 1 mm apart) that were purged with argon and left to polymerize at room temperature for 3 hr under an argon environment prior to use.

Example 12: Preparation of 10%M 2%X AAm/BIS hydrogel cassettes for gel electrophoresis using 25% aqueous ethylene glycol as solvent

10%M 2%X solutions (10 g) were prepared by dissolving AAm (978.3 mg) and BIS (21.7 mg) in ethylene glycol (2.7 g) and water (3.8 g). 1.5M Tris-HCl buffer (pH 8.8, 2.5 g). The stock buffer solution was prepared by dissolving Tris (27.23 g) in water (80 ml) and adjusted to the pH of 8.8 with 6 N HCl followed by making up the required volume (150 ml) with water.

The monomer solution was degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS (64.1 μ m) and 10% (v/v) TEMED (42.4 μ m). The gel solution (7 ml) was then immediately cast between two glass plates (8 x 8 cm, 1 mm apart) that were purged with argon and left to polymerize at room temperature for 3 hr under an argon environment prior to use.

Example 13: Preparation of 10%M 2%X AAm/BIS hydrogel cassettes for gel electrophoresis using 25% aqueous propylene glycol as solvent

10 10%M 2%X solutions (10 g) were prepared by dissolving AAm (978.3 mg) and BIS (21.7 mg) in propylene glycol (2.7 g) and water (3.8 g). 1.5M Tris-HCl buffer (pH 8.8, 2.5 g). The stock buffer solution was prepared by dissolving Tris (27.23 g) in water (80 ml) and adjusted to the pH of 8.8 with 6 N HCl followed by making up the required volume (150ml) with water.

15 The monomer solution was degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS (64.1 μ m) and 10% (v/v) TEMED (42.4 μ m). The gel solution (7 ml) was then immediately cast between two glass plates (8 x 8 cm, 1 mm apart) that were purged with argon and left to polymerize at room temperature for 3 hr under an argon environment prior to use.

Example 14: Preparation of 10%M 2%X AAm/BIS hydrogel cassettes for gel electrophoresis using 11% aqueous tri(ethylene glycol) as solvent

25 10%M 2%X solutions (10 g) were prepared by dissolving AAm (978.3 mg) and BIS (21.7 mg) in tri(ethylene glycol) (1.2 g) and water (5.3 g). 1.5M Tris-HCl buffer (pH 8.8, 2.5 g). The stock buffer solution was prepared by dissolving Tris (27.23 g) in water (80 ml) and adjusted to the pH of 8.8 with 6 N HCl followed by making up the required volume (150 ml) with water.

30 The monomer solution was degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS (64.1 μ m) and 10% (v/v) TEMED (42.4 μ m). The gel solution (7 ml) was then immediately cast between two glass plates (8 x 8 cm, 1 mm apart) that were purged with argon and left to polymerize at room temperature for 3 hr under an argon environment prior to use.

Example 15: Preparation of 10%M 2%X AAm/BIS hydrogel cassettes for gel electrophoresis using 5.5 and 11% aqueous poly(ethylene glycol) 400 as solvent

10%M 2%X solutions (10 g) were prepared by dissolving AAm (978.3 mg) and BIS (21.7 mg) in poly(ethylene glycol) 400 (0.6 or 1.2 g) and water (5.3 or 5.9g). 1.5M Tris-HCl buffer (pH 8.8, 2.5 g). The stock buffer solution was prepared by dissolving Tris (27.23 g) in water (80 ml) and adjusted to the pH of 8.8 with 6 N HCl followed by making up the required volume (150 ml) with water.

The monomer solution was degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS (64.1 μ m) and 10% (v/v) TEMED (42.4 μ m). The gel solution (7 ml) was then immediately cast between two glass plates (8 x 8 cm, 1 mm apart) that were purged with argon and left to polymerize at room temperature for 3 hr under an argon environment prior to use.

15 EVALUATION OF ACRYLAMIDE HYDROGELS

Swelling tests

Gels made according to examples 2-6 were immersed in water (500 g) for 1 week during which the immersing solution (water) was exchanged on a daily basis. The gel was then dried in a 40°C oven for 1 week. The equilibrium solvent content of the gel was determined by the following equation.

$$\text{Equilibrium solvent content (ESC)} = \frac{\text{weight(swollen gel)} - \text{weight(dried gel)}}{\text{weight(dried gel)}}$$

25 Example 16: ESC (water) of AAm/BIS hydrogels synthesized in water and aqueous solutions of ethylene glycol

Polymerization Solvent	ESC
water	12.1
25% ethylene glycol / 75% water	14.3
50% ethylene glycol / 50% water	15.4
75% ethylene glycol / 25% water	20.0

Example 17: ESC (water) of AAm/BIS hydrogels synthesized in water and aqueous solutions of propylene glycol

Polymerization Solvent	ESC
water	12.1
25% propylene glycol / 75% water	15.3
50% propylene glycol / 50% water	21.9
75% propylene glycol / 25% water	28.6

5

Example 17: ESC (water) of AAm/BIS hydrogels synthesized in water and aqueous solutions of tri(ethylene glycol)

Polymerization Solvent	ESC
water	12.1
11% tri(ethylene glycol) / 89% water	12.7
22% tri(ethylene glycol) / 78% water	14.5
33% tri(ethylene glycol) / 66% water	16.3
44% tri(ethylene glycol) / 56% water	18.1
55% tri(ethylene glycol) / 45% water	21.8
61% tri(ethylene glycol) / 39% water	25.0
64% tri(ethylene glycol) / 36% water	26.3
66% tri(ethylene glycol) / 34% water	26.7
69% tri(ethylene glycol) / 31% water	30.0
72% tri(ethylene glycol) / 28% water	32.8

Example 17: ESC (water) of AAm/BIS hydrogels synthesized in water and aqueous solutions of poly(ethylene glycol) 400

Polymerization Solvent	ESC
water	12.1
6% poly(ethylene glycol) 400 / 94% water	13.2
11% poly(ethylene glycol) 400 / 89% water	14.0
16% poly(ethylene glycol) 400 / 84% water	15.2
22% poly(ethylene glycol) 400 / 78% water	17.3

5 Turbidity measurements .

The turbidity of gels made according to examples 7-9 was measured using UV-visible spectrophotometry. Distilled water was used for the baseline and the absorbance of each gel sample and the corresponding polymerization solvent were recorded at 100nm intervals between 300 and 800 nm. The turbidity of the gel samples were determined by the following equation.

$$\text{Turbidity} = -\log_e(10^{-(\text{absorbance of gel} - \text{absorbance of polymerization solvent})})$$

Example 18: Turbidity of 10%M 2%X AAm/BIS hydrogels synthesized in water and aqueous solutions of poly(ethylene glycol) 400 at 500 nm (room temperature and 40°C)

Polymerization Solvent	Turbidity (Room Temperature)	Turbidity (40°C)
Water	0	0
6% poly(ethylene glycol) 400 / 94% water	0	0
11% poly(ethylene glycol) 400 / 89% water	0	0
16% poly(ethylene glycol) 400 / 84% water	0.23	0
19% poly(ethylene glycol) 400 / 81% water	0.46	0.18
22% poly(ethylene glycol) 400 / 78% water	1.27	0.32
27% poly(ethylene glycol) 400 / 73% water	6.90	5.4
33% poly(ethylene glycol) 400 / 66% water	8.06	7.5

5 *Visual opacity corresponds to a turbidity value of > 0.3 at 500nm

Example 19: Turbidity of 10%M.2%X AAm/BIS hydrogels synthesized in water and aqueous solutions of tri(ethylene glycol), poly(ethylene glycol) 400 and poly(ethylene glycol) 20,000 at 500 nm (room temperature)

10 Turbidity testing showed that the onset of opacity occurs at 72%, 19% and 0.1% for aqueous solution of tri(ethylene glycol), poly(ethylene glycol) 400 and poly(ethylene glycol) 20,000 respectively.

Gel Electrophoresis

15 Standard SDS-PAGE was performed on the acrylamide hydrogel cassette (example 11-15) using a constant voltage of 150 V and Tris-glycine electrophoresis running buffer. The electrophoresis running buffer (100 ml) was prepared by dissolving Tris (9 g), SDS (3 g), and glycine (43.2 g) in water and diluting 1:5 with water before use. 10 µL of Kaleidoscope pre-stained protein marker or SDS-PAGE molecular weight standards (broad range) was syringed into sample wells and separated. Gels with SDS-PAGE molecular weight standards (broad range) were stained for 3 hr using Coomassie

20

Blue solution and de-stained overnight with 10% aqueous acetic acid. The migration ratio of a protein was determined by the following equation.

$$\text{Migration Ratio} = \frac{\text{distance travelled by protein}}{\text{distance travelled by dye front}}$$

5

Kaleidoscope Prestained Standards (Bio-Rad 161-0324)

Protein	Calibrated MW
Myosin	206,000
β -galactosidase	128,000
Bovine serum albumin	81,000
Carbonic anhydrase	40,300
Soybean trypsin inhibitor	31,600
Lysozyme	19,300
Aprotinin	7,800

SDS-PAGE Molecular Weight Standards (broad range, Bio-Rad 161-0317)

Protein	Calibrated MW
Myosin	200,000
β -galactosidase	116,250
Phosphorylase b	97,400
Serum albumin	66,200
Ovalbumin	45,000
Carbonic anhydrase	31,000
Trypsin inhibitor	21,500
Lysozyme	14,400
Aprotinin	6,500

Example 20: Electrophoresis of 10%M 2%X AAm/BIS gel cassette synthesized in water and aqueous solutions of ethylene glycol or propylene glycol

Migration ratios of Kaleidoscope Pre-stained Standards in 10%M 2%X acrylamide gel cassette synthesized in water, aqueous solutions of ethylene glycol (25%) or propylene glycol (25%) are shown in Figure 1.

Example 21: Electrophoresis of 10%M 2%X acrylamide gel cassette synthesized in water and aqueous solutions of poly(ethylene glycol) 400

Migration ratios of SDS-PAGE Molecular Weight Standards (board range) in 10%M 2%X acrylamide gel cassette synthesized in water, or aqueous solutions of poly(ethylene glycol) are shown in Figure 2.

Example 22: Electrophoresis of 10%M 2%X AAm/BIS gel cassette synthesized in water and aqueous solutions of tri(ethylene glycol) or poly(ethylene glycol) 400

Migration ratios of SDS-PAGE Molecular Weight Standards (board range) in 10%M 2%X acrylamide gel cassette synthesized in water or aqueous solutions of tri(ethylene glycol) and poly(ethylene glycol) are shown in Figure 3.

Example 23: Electrophoresis of 10%M 2%X AAm/BIS gel cassette synthesized in water and aqueous solutions of tri(ethylene glycol)

Migration ratios of Kaleidoscope Prestained Standards in 10%M 2%X acrylamide gel cassette synthesized in water and aqueous solutions of tri(ethylene glycol) are shown in Figure 4.

25 Preparation of Methacrylamide Hydrogels

Example 24: Preparation of 10%M 2%X methacrylamide/N,N'-methylenebismethacrylamide hydrogels using aqueous glycerol as solvent

Aqueous solution of glycerol (75%) were prepared by mixing appropriate amount of water and glycerol. methacrylamide (978.6 mg) and N,N'-methylenebismethacrylamide (21.4 mg) were added to the above solutions (9 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond)

composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment to produce a hydrogel that was visually clear.

5 Opacity and reduction in mechanical integrity was observed when the above methacrylamide hydrogel was equilibrated in water.

Preparation of 2-Hydroxyethyl Acrylate (HEA) Hydrogels

Example 25: Preparation of HEA/EGDA hydrogels using water as solvent

10 10%M HEA hydrogels at 3, 4, 5, 6, and 10%X were prepared by mixing the appropriate amount of HEA, EGDA. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment.

15 All of the resultant polymers were not visually clear, the opacity was observed to increase with increasing %X.

Example 26: Preparation of 10%M 6.5%X HEA/EGDA hydrogels using aqueous ethylene glycol as solvent

20 Aqueous solutions of ethylene glycol (20, 40, 60 and 80%) were prepared by varying amounts of ethylene glycol and water. HEA (951.5 mg) and EGDA (48.5 mg) were added to the above solutions (9 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10%
25 (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment.

30 The polymers synthesized in 0 and 20% ethylene glycol solutions were opaque. The polymer synthesized in 40% ethylene glycol solution was slightly opalescence. The polymer synthesized in 60 and 80% ethylene glycol solutions were visually clear and remained visually clear after equilibration in water.

Example 27: Preparation of 10%M 6.5%X HEA/EGDA hydrogels using aqueous solutions of poly(ethylene glycol) 200, tetrahydrofuran, or methanol as solvent

60% aqueous solutions of PEG 200, tetrahydrofuran, or methanol were prepared. HEA (951.5 mg) and EGDA (48.5 mg) were added to the above solutions (9 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment.

The polymers synthesized in 60% PEG 200, 60% tetrahydrofuran, and 60% methanol were visually clear. All gels were visually clear and remained visually clear after equilibration in water.

Preparation of 2-Hydroxyethyl Methacrylate (HEMA) Hydrogels

Example 28: Preparation of 5%X HEMA/EGDMA hydrogels using water as solvent

10%, 20%, 30% and 40%M HEMA hydrogels were prepared by mixing the appropriate amount of HEMA, EGDMA and water (10 g total) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment.

All of the resultant polymers were highly opaque and had little mechanical strength.

Example 29: Preparation of 15%M 5%X HEMA/EGDMA hydrogels for turbidity measurements using aqueous ethylene glycol, tri(ethylene glycol), PEG 400 or PEG 6,000 as solvent

Aqueous solutions of ethylene glycol, tri(ethylene glycol), PEG 400 or PEG 6,000 (40, 45, 50, 60 and 70%) were prepared. HEMA (1.442 g) and EGDMA (57.8 mg) were added to the above solutions (8.5 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l samples were pipetted into disposable cuvettes (10 x 10

x 45 mm³) and the polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 30: Preparation of 15%M HEMA/EGDMA hydrogels for turbidity measurements using 50% PEG 200 as solvent

Aqueous solution of PEG 200 (50%) was prepared by mixing the appropriate amount of PEG 200 and water. 15%M HEMA hydrogels with 0, 2.5, 5, 7.5 and 10%X were prepared by mixing the appropriate amounts of HEMA, EGDMA and 50% PEG solution (10 g total) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 31: Preparation of 5%X HEMA/EGDMA hydrogels for turbidity measurements using 50% PEG 200 as solvent

Aqueous solution of PEG 200 (50%) was prepared by mixing the appropriate amount of PEG 200 and water. 5%X HEMA hydrogels with 7.5, 10, 12.5, 15, 20, 40%T were prepared by mixing the appropriate amounts of HEMA, EGDMA and 50% PEG solution (10 g total) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 32: Preparation of 15%M 5%X HEMA/EGDMA hydrogels for turbidity measurements using aqueous propylene glycol, tri(propylene glycol) or PPG 425 as solvent

Aqueous solutions of propylene glycol, tri(propylene glycol) or PPG 425 (30, 35, 40, 45 and 50%) were prepared. HEMA (1.442 g) and EGDMA (57.8 mg) were added to the above solutions (8.5 g) in disposable glass vials. The monomer solution was then

degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 33: Preparation of 15%M 5%X HEMA/EGDMA hydrogels for turbidity measurements using aqueous PEG dimethyl ether 500 as solvent

Aqueous solutions of PEG dimethyl ether 500 (30, 35, 40, 45 and 50%) were prepared. HEMA (1.442 g) and EGDMA (57.8 mg) were added to the above solutions (8.5 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 34: Preparation of 15%M 5%X HEMA/EGDMA hydrogels for turbidity measurements using aqueous ethylene glycol monomethyl ether, ethylene glycol monoethyl ether or ethylene glycol monobutyl ether as solvent

Aqueous solutions of ethylene glycol monomethyl ether, ethylene glycol monoethyl ether or ethylene glycol monobutyl ether (30, 35, 40, 45 and 50%) were prepared. HEMA (1.442 g) and EGDMA (57.8 mg) were added to the above solutions (8.5 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 35: Preparation of 15%M 5%X HEMA/EGDMA hydrogels for turbidity measurements using aqueous poly(ethylene glycol - co - propylene glycol) 2,500 (poly(eg-co-pg) 2,000), poly(ethylene glycol - co - propylene glycol) 12,000 (poly(eg-co-pg) 12,000) or poly(ethylene glycol - block - propylene glycol - block - ethylene glycol) 1,900 (poly(eg-b-pg-eg) 1,900) as solvent

Aqueous solutions of poly(eg-co-pg) 2,000, poly(eg-co-pg) 12,000 or poly(eg-b-pg-eg) 1,900 (30, 35, 40, 45 and 50%) were prepared. HEMA (1.442 g) and EGDMA (57.8 mg) were added to the above solutions (8.5 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 36: Preparation of 15%M 5%X HEMA/EGDMA hydrogels for turbidity measurements using aqueous PEG 400 or PPG 425 as solvent

Aqueous solutions of PEG 400 or PPG 425 (30, 50, 70 and 90%) were prepared. HEMA (1.442 g) and EGDMA (57.8 mg) were added to the above solutions (8.5 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 37: Preparation of 15%M 5%X HEMA/EGDMA hydrogels for turbidity measurements using aqueous solutions of poly(eg-b-pg-b-eg) 1900 and PEG 400 mixtures as solvent

40% aqueous solutions of poly(eg-b-pg-b-eg) 1900 and PEG 400 mixtures (0, 12.5, 25, 50, 75, 87.5 and 100% poly(eg-b-pg-b-eg) 1900) were prepared. HEMA (1.442 g) and EGDMA (57.8 mg) were added to the above solutions (8.5 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l

samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the polymerization was then allowed to proceed at room temperature overnight under an argon environment.

5 **Example 38: Preparation of 15%M 5%X HEMA/EGDMA hydrogels for turbidity measurements using aqueous solutions of ethylene glycol monomethyl ether and PEG 200 mixtures as solvent**

35% aqueous solutions of ethylene glycol monomethyl ether and PEG 200 mixtures (0, 14, 28, 57, 86 and 100% ethylene glycol monomethyl ether) were prepared. 10 HEMA (1.442 g) and EGDMA (57.8 mg) were added to the above solutions (8.5 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 µl samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the 15 polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 39: Preparation of 5%X HEMA/EGDMA hydrogels for swelling tests using aqueous tri(ethylene glycol) as solvent

20 Aqueous solution of tri(ethylene glycol) (60%) were prepared. 20, 40, 60 and 80%M HEMA/EGDMA hydrogels were prepared by mixing the appropriate amount of HEMA, EGDMA and the above 60% tri(ethylene glycol) solution in disposable glass vials (10 g total). The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of 25 freshly made up 10% (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 40: Preparation of 10%M 5%X HEMA/EGDMA hydrogels for swelling tests using water and aqueous solutions of PEG 200 or PEG 4000 as solvent

30 Aqueous solutions of PEG 200 or PEG 4000 (50%) were prepared. HEMA (1.442 g) and EGDMA (57.8 mg) were added to the above solutions (8.5 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond)

composed of freshly made-up 10% (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment.

5 **Example 41: Preparation of 15%M 4%X HEMA/EGDMA membrane for electrophoretic separation analysis using aqueous solutions of PEG 200 as solvent**

Unwoven poly(ethyleneterephthalate) (PET) sheets that served as a mechanical support were treated with aqueous solution of Teric BL8 (0.5% (v/v)), Huntsman Corp.
10 Australia) a non-ionic surfactant used to improve surface wettability.

Aqueous solution of PEG 200 (80%) were prepared. 15%M 4%X HEMA/EGDMA mixtures with the above PEG 200 solution were polymerized into thin membranes with Teric BL8 treated unwoven PET sheet as the supporting substrate.

15 **Evaluation of HEMA Hydrogels**

Turbidity testing

All HEMA hydrogels which were visually clear after the synthesis remained visually clear after the solvent was exchanged with water.

20 **Example 42: Turbidity of 15%M 5%X HEMA/EGDMA hydrogels synthesized in aqueous ethylene glycol, tri(ethylene glycol), PEG 400 or PEG 6,000 solutions at 500 nm**

Turbidity results of polymers synthesized according to Example 29 are shown in Figure 5.

25

Example 43: Turbidity of 15%M HEMA/EGDMA hydrogels synthesized in 50% aqueous PEG 200 solution at 500nm

Turbidity results of polymers synthesized according to Example 30 are shown in Figure 6.

Example 44: Turbidity of 5%X HEMA/EGDMA hydrogels synthesized in 50% aqueous PEG 200 solution at 500nm.

Turbidity results of polymers synthesized according to Example 31 are shown in Figure 7.

5

Example 45: Turbidity of 15%M 5%X HEMA/EGDMA hydrogels synthesized in aqueous propylene glycol, tri(propylene glycol) or PPG 425 as solvent

Turbidity results of polymers synthesized according to Example 32 are shown in Figure 8.

10

Example 46: Turbidity of 15%M 5%X HEMA/EGDMA hydrogels synthesized in aqueous PEG dimethyl ether 500 solutions.

Turbidity results of polymers synthesized according to Example 33 are shown in Figure 9.

15

Example 47: Turbidity of 15%M 5%X HEMA/EGDMA hydrogels synthesized in aqueous ethylene glycol monomethyl ether, ethylene glycol monoethyl ether or ethylene glycol monobutyl ether as solvent

Turbidity results of polymers synthesized according to Example 34 are shown in Figure 10.

20

Example 48: Turbidity of 15%M 5%X HEMA/EGDMA hydrogels synthesized in aqueous poly(ethylene glycol - co - propylene glycol) 2,500 (poly(eg-co-pg) 2,000), poly(ethylene glycol - co - propylene glycol) 12,000 (poly(eg-co-pg) 12,000) or poly(ethylene glycol - block - propylene glycol - block - ethylene glycol) 1,900 (poly(eg-b-pg-eg) 1,900) as solvent

25

Turbidity results of polymers synthesized according to Example 35 are shown in Figure 11.

Example 49: Turbidity of 15%M 5%X HEMA/EGDMA hydrogels synthesized in aqueous PEG 400 or PPG 425 as solvent

Turbidity results of polymers synthesized according to Example 36 are shown in Figure 12.

5

Example 50: Turbidity of 15%M 5%X HEMA/EGDMA hydrogels synthesized in aqueous solutions of poly(eg-b-pg-b-eg) 1900 and PEG 400 mixtures as solvent

Turbidity results of polymers synthesized according to example 37 are shown in Figure 13.

10

Example 51: Turbidity of 15%M 5%X HEMA/EGDMA hydrogels synthesized in aqueous solutions of ethylene glycol monomethyl ether and PEG 200 mixtures as solvent

Turbidity results of polymers synthesized according to Example 38 are shown in Figure 14.

15

Example 52: Swelling test (water) of 5%X HEMA/EGDMA hydrogels at 20, 40, 60, 80%M synthesized in 60% aqueous tri(ethylene glycol) solution

Hydrogel	ESC (water)
20%M 5%X	0.81
40%M 5%X	0.72
60%M 5%X	0.56
80%M 5%X	0.54

20

Example 53: Swelling test (water) of 15%M 5%X HEMA/EGDMA hydrogels synthesized in 50% aqueous solutions of PEG 200 or PEG 4000

ESC(water) for 15%M 5%X HEMA/EGDMA hydrogel synthesized in 50% PEG 200 solution was found to be 0.65.

25. ESC(water) for 15%M 5%X HEMA/EGDMA hydrogel synthesized in 50% PEG 4000 solution was found to be 0.83.

Example 54: Swelling test (40% aqueous solutions of ethylene glycol, PEG 600, PEG 4000 or PEG 6000) of 15%M 5%X HEMA/EGDMA hydrogels synthesized in 50% aqueous solutions of PEG 200 or PEG 400

5 Hydrogels prepared in Example 40 were immersed in water (500 g) for 1 week during which the immersing solution (water) was exchanged on a daily basis. The gel was then dried in a 40°C oven for 1 week. The dried gels were then immersed in 50% aqueous solutions of ethylene glycol, PEG 600, PEG 4000 or PEG 6000) for 1 week during which the immersing solution was exchanged on a daily basis. The ESC of the
10 gels are shown in the following table.

	ESC (40% EG)	ESC (40% PEG 600)	ESC (40% PEG 4000)	ESC (40% PEG 6000)
15%M 5%X hydrogels synthesized in 50% PEG 200	0.98	2.99	1.31	1.14
15%M 5%X hydrogels synthesized in 50% PEG 4000	1.30	3.48	3.00	2.45

Example 55: Electrophoresis separation analysis of 15%M 4%X HEMA/EGDMA membrane synthesized in 80% aqueous PEG 200 solution

15 Samples of known molecular weight and size were run through a Gradiflow™ BF 200 unit to investigate the relative pore size formed in HEMA hydrogel networks. The protein standards were placed in a buffer solution and run by current from the stream 1 section of the unit above the membrane. Proteins smaller than the pores of the
20 membrane will pass through the membrane into the stream 2 section of the unit. The larger proteins will be recycled back into the *stream 1* section. Ten µl samples from both the two streams of the unit are taken every 10 minutes and detected using SDS-PAGE. The migration pattern should indicate what sized samples passed through the membrane. More details on the construction and operation of this unit can be found in US 5650055, US 5039386, WO 00/56792 and WO 00/13776, incorporated herein by
25 reference.

The separation and migration pattern of Bovine serum albumin (MW 67,000) by a 15%M 4%X HEMA/EGDMA membrane synthesized in 80% aqueous PEG 200 solution (Example 41) using 40 mM MES bis-TRIS buffer is shown in Figure 15.

5 ***Preparation of Poly(ethylene glycol) Methacrylate (HEMA) hydrogels***

Example 56: Preparation of 15%M 5%X PEGMA 526/EGDMA hydrogels for turbidity measurements using aqueous PEG 400 or PPG 425 as solvent

Aqueous solutions of PEG 400 or PPG 425 (0, 30, 50 and 70%) were prepared. PEGMA (1.485 g) and EGDMA (14.7 mg) were added to the above solutions (8.5 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. Two 375 μ l samples were pipetted into disposable cuvettes (10 x 10 x 45 mm³) and the polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 57: Turbidity of 15%M 5%X PEGMA 526/EGDMA hydrogels synthesized in aqueous PEG 400 or PPG 425 as solvent

Turbidity results of polymers synthesized according to Example 56 are shown in Figure 16.

Preparation of optically clear hydrogels

Example 58: ¹³C NMR relaxation measurements of acrylamide hydrogels

Monomer solutions (2 g) were prepared by dissolving AAm and Bis in the appropriate amount of D₂O (10% TMSPA-Na, 0.2g), water and PEG-400. The monomer solution was then degassed by argon purging prior to the addition of the initiator system composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED (0.05 mol% initiator per double bond). This mixture was immediately pipetted into 5 mm NMR tube (0.38 mm wall thickness) and the polymerization was allowed to proceed at room temperature overnight under an argon environment.

¹³C NMR spectra were obtained using a Varian Unity Plus 400 spectrometer operating at 100 MHz. Spin-lattice relaxation times (T₁) were measured by the inversion-recovery method at 25°C. Recycled delays were set to 7s (>3T₁), with delay times (τ) of

10, 50, 100, 200, 300, 400, 500, 600, 700, 800, and 1000 ms. The T_1 parameters were calculated by fitting the data to the following equation.

$$I(\tau) = I(\tau=0) (1 - 2 \exp(-\tau/T_1)) \dots (4)$$

when I is the intensity of the transformed peaks.

5

Example 59: Real-time viscosity measurements of acrylamide polymerizations

Monomer solutions (200 g) were prepared by dissolving AAm and Bis in the appropriate amount of water and PEG-400. The monomer solution was then degassed by argon purging prior to the addition of the initiator system composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED (0.05 mol% initiator per double bond). The viscosity of the reaction mixture was measured by a Brookfield® DV-II+ viscometer (0.3 rpm, LV-3 spindle). The experiments were performed in a glove box with controlled oxygen levels (< 0.1 % O₂).

Example 60: Preparation of acrylamide hydrogels for swelling studies

Monomer solution (10 g) was prepared by dissolving AAm and Bis in an appropriate amount of water and PEG-400 in disposable glass vials. The monomer solution was then degassed by argon purging prior to the addition of the initiator system (0.2 mol% initiator per double bond), composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment.

Example 61: Kinetic swelling studies of acrylamide hydrogels

The gel made according to the above procedure was immersed in water (500 g) for 1 week during which the immersing solution (water) was exchanged on a daily basis. The gel was then dried in a 40°C oven for 1 week and re-swelled in water. The weight of the swollen gel was continuously monitored for 48 hours.

ESC of the gel was determined by the following equation.

$$\text{Equilibrium solvent content (ESC)} = \frac{\text{weight(swollen gel)} - \text{weight(dried gel)}}{\text{weight(dried gel)}}$$

30

Example 62: Preparation of 15%M 5%X HEMA/EGDMA hydrogels using aqueous ethylene glycol monomethyl ether as solvent

5 Aqueous solutions of ethylene glycol monomethyl ether (80, 85 and 90%) were prepared. HEMA (1.442 g) and EGDMA (57.8 mg) were added to the above solutions (8.5 g) in disposable glass vials. The monomer solution was then degassed by argon purging prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. The polymerization was then allowed to proceed at room temperature overnight under an argon environment. All resultant gels were visually clear.

10

Example 63: ^{13}C T_1 (25°C, 100MHz) for 20%M 2%X acrylamide hydrogels synthesized in the presene of various amount of PEG-400.

% PEG-400	T_1 (α -carbon)	T_1 (β -carbon)	T_1 (carbonyl)
2.5	240	125	1330
7.5	240	135	1350
12.5	261	140	1400
17.5	270	155	1400
22.5	340	180	1730
27.5	420	230	2185

Example 64: ESC (water) of AAm/BIS hydrogels from kinetic swelling studies

% PEG-400	7.5	12.5	17.5	22.5	27.5
Time(hr)					
0.5	3.14	3.34	3.34	2.99	2.70
1	3.52	3.82	3.81	3.34	2.99
1.5	3.81	4.14	4.18	3.59	3.23
2	4.05	4.44	4.48	3.81	3.42
3	4.47	4.97	5.10	4.20	3.76
4	4.86	5.55	5.50	4.54	4.05
5	5.23	6.03	6.04	4.93	4.42
24	12.04	13.16	13.02	9.3	6.84
48	15.22	16.40	16.53	13.21	8.58

5 SUMMARY

Examples 2 to 23 show that the following:

Acrylamide hydrogels can undergo polymerization-induced phase separation when it is synthesized in solvents containing poly(ethylene glycol) with 3 repeating units or more.

- 10 Turbidity testing showed that the onset of opacity (i.e. phase separation) occurs at lower concentrations of poly(ethylene glycol) with increasing molecular weight of poly(ethylene glycol).

- 15 Acrylamide hydrogels synthesized in the presence of water-soluble entities have in general, larger pores than those synthesized in water. Such gels however cannot be synthesized in solvents containing high concentrations of poly(ethylene glycol) with high molecular weight.

It is well known that when methacrylamide is polymerized in water, an opaque polymer mass is obtained. Example 24 showed that visually clear hydrogels can be obtained from methacrylamide by using hydro-organic solution as the polymerization

solvent. Such hydrogels, however, became opaque and lost their mechanical integrity when the organic solvent was subsequently exchanged with water. This demonstrated that although by using a hydro-organic solution as the polymerization solvent, a visually clear hydrogel can be obtained from monomers that produce water-immiscible polymers,
5 many of the resultant hydrogels cannot be used in aqueous media.

Examples 25-27 show that:

HEA hydrogels that are synthesized using water as solvent are opaque and have poor mechanical integrity.

10 Visually clear HEA hydrogels can be synthesized by careful selection of water-miscible entities. Such gels remained visually clear after the water-miscible entities were exchanged with water. This contrasts with the teaching from prior art observations made in methacrylamide hydrogels.

Examples 28-37 and 42-51 show that:

15 HEMA hydrogels that are synthesized in water are opaque and have poor mechanical integrity.

Visually clear HEMA hydrogels can be synthesized by careful selection of water-miscible entities. Such gels remained visually clear after the water-miscible entities were exchanged with water.

HEMA hydrogels have very different behaviour to acrylamide hydrogels.
20 Polymerization-induced phase separation occurs at low concentrations of water-miscible entities (e.g. poly(ethylene glycol)), and the gels become more visually clear and the mechanical properties of such gels increases when the concentrations of water-miscible entities increases. This contrasts with prior art acrylamide hydrogels, which state that high concentrations of water-miscible entities would lead to phase separations.

25 Unexpectedly, turbidity testing shows that in contrast to acrylamide hydrogels, poly(ethylene glycol) with higher molecular weight improves the visual and mechanical properties of the resultant gel (Figure 5). This contrasts with prior art acrylamide systems, which state that water-miscible entities with high molecular weight would lead to phase separation.

30 Figure 7 (Example 31 and 44) shows that visually clear HEMA hydrogels can be obtained from reaction mixtures with low initial monomer concentrations. This contrasts with prior art HEMA gels.

Figure 8 and 12 (Example 32, 36 and 45, 49) demonstrate the usage of poly(propylene glycol) as water-miscible entities. The usage of poly(propylene glycol) has not been reported in the literature on hydrogel synthesis.

5 Figures 9 and 10 (Example 33-34 and 46-47) demonstrate the usage of poly(ethylene glycol) derivatives (i.e. alkyl ether) as water-miscible entities. The usage of such derivatives has not been reported in the literature on acrylamide hydrogel synthesis.

10 Figure 11 (Example 35 and 48) demonstrate the usage of random and block copolymers of poly(ethylene glycol) and poly(propylene glycol) as water-miscible entities. The usage of such water-miscible entities has not been reported previously.

Figures 13 and 14 (Example 36-37 and 49-51) demonstrate the usage of two different types of water-miscible entities together in the same solvent system. The usage of such mixtures of water-miscible entities have not been reported previously.

15 Example 52 shows that by careful selection of the water-miscible entities, HEMA hydrogels with high water swelling properties can be formed from monomer mixtures with low monomer concentrations (i.e. <50%M). It also shows the increase in water swelling properties with decreasing total monomer concentrations. This contrasts with the prior which states the opposite.

20 Examples 52 and 53 show that water swelling properties of HEMA hydrogels are dependent upon the initial monomer concentration, the types of water-miscible entities and the concentration of water-miscible entities.

Example 53 further demonstrates that the water swelling properties of the hydrogels increases when the molecular weight of the water-miscible entities (i.e. poly(ethylene glycol)) is increased.

25 Example 54 shows the swelling properties of two different hydrogels. Hydrogel A was synthesized in the presence of a water-miscible entity with low molecular weight; hydrogel B was synthesized in the presence of a water miscible entity with high molecular weight.

30 Swelling of Hydrogel A and B in mixtures composed of water and organic solvents with different molecular weight shows that:

Hydrogel B swells more in all solvents.

Hydrogel A has low swelling properties in solvents with organic solvents with high molecular weight.

Hydrogel B has significantly higher swellings in solvents with high molecular weight than Hydrogel A.

5 The above observations show that as the molecular weight of the water-miscible entities increases, the pore size of the gels become dependent upon the size of the water-miscible entities. Such gels have macroporous pores and hence are able to swell more in solvents with high molecular weight solutes, because of the increased diffusion of organic solvent with high molecular weights into the gel.

10 Examples 56 and 57 demonstrate the usages of poly(ethylene glycol) and poly(propylene glycol) as water-miscible entities in other hydrogels prepared from α,ω -(meth)acryloyloxy monomers. Poly(ethylene glycol) methacrylate was used in these examples. The present invention extends to derivatives of HEMA and HEA, that is, monomers with the same (meth)acrylate ester structure with HEMA and HEA, but different side chains.

15 Example 58 and 63 show that PIPS occur in 20%M 2%X acrylamide hydrogels synthesized in the presence of 22.5 and 27.5% PEG-400, but can be avoided by the careful selection of the polymerization solvent. It is therefore possible to prepare visually clear hydrogels even when the polymerization solvent is immiscible with the corresponding linear polymer analogues.

20 Figure 17 is a schematic diagram of the formation process of 20%M acrylamide hydrogel, it demonstrates the relationship between the 'freezing concentration' of the reaction mixture, the phase boundary, and the concentration and properties of the water-miscible entity which alter the region of immiscibility on the diagram.

25 Example 59 and Figure 18 demonstrate the relationship between the 'freezing concentration' of the reaction mixture and the phase boundary, it can be seen that visually clear gels can be obtained. In systems where the 'freezing concentration' of the reaction mixtures is reached before the onset of PIPS.

Examples 60, 61, and 64 show that hydrogels prepared by the approach of this invention have superior swelling properties to that prepared by systems that reaches the phase boundary before the gel point (22.5 and 27.5% PEG-400).

30 Example 62 shows that by using a mixture of water and water-miscible entities as the polymerization solvent, visually clear HEMA hydrogels can be prepared even when the polymerization solvent is immiscible with the corresponding linear polymer analogues which are water immiscible.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this 14th day of May 2003

The University of Melbourne

Patent Attorneys for the Applicant:

ALLENS ARTHUR ROBINSON

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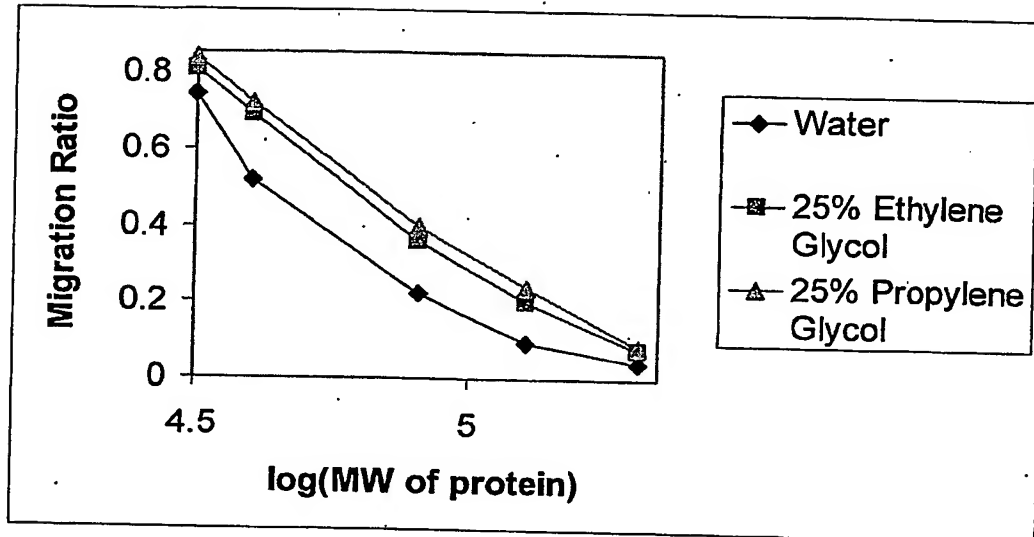


Figure 1

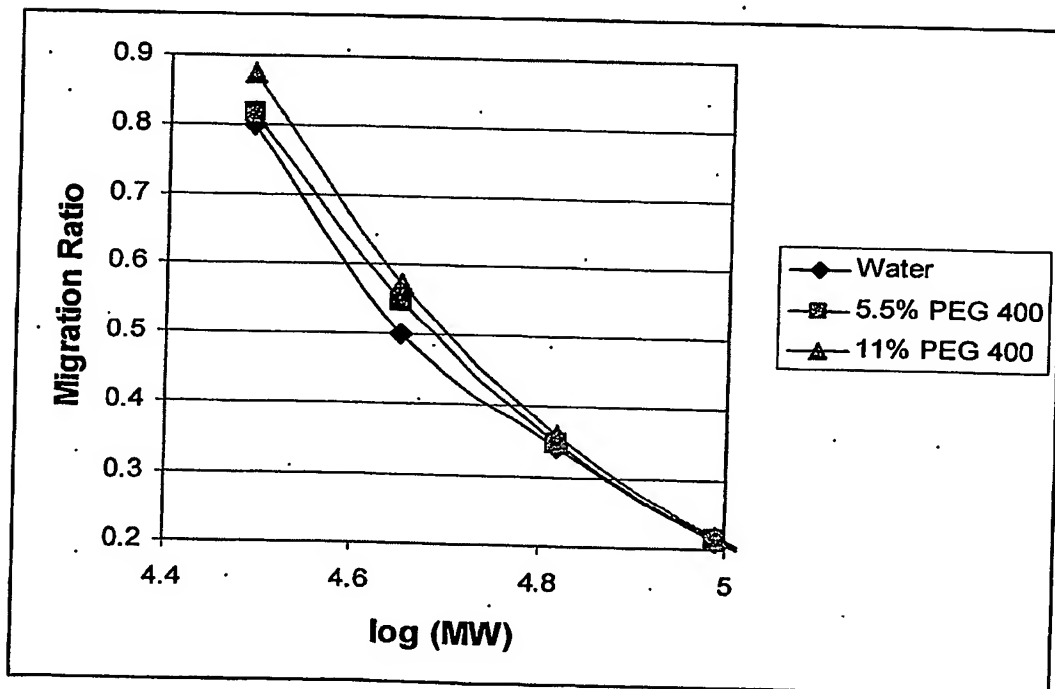


Figure 2

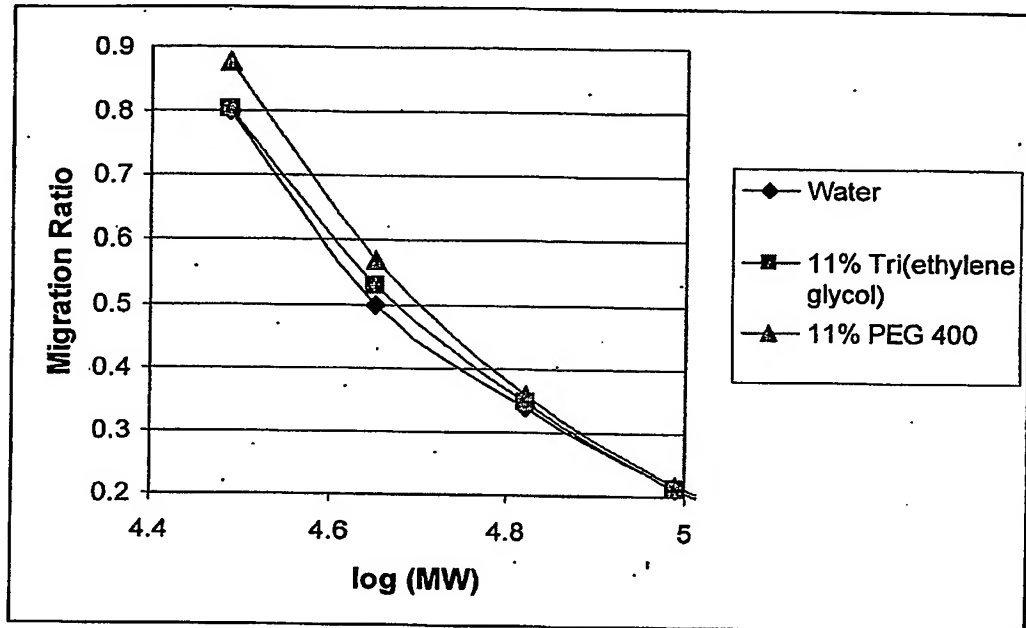


Figure 3

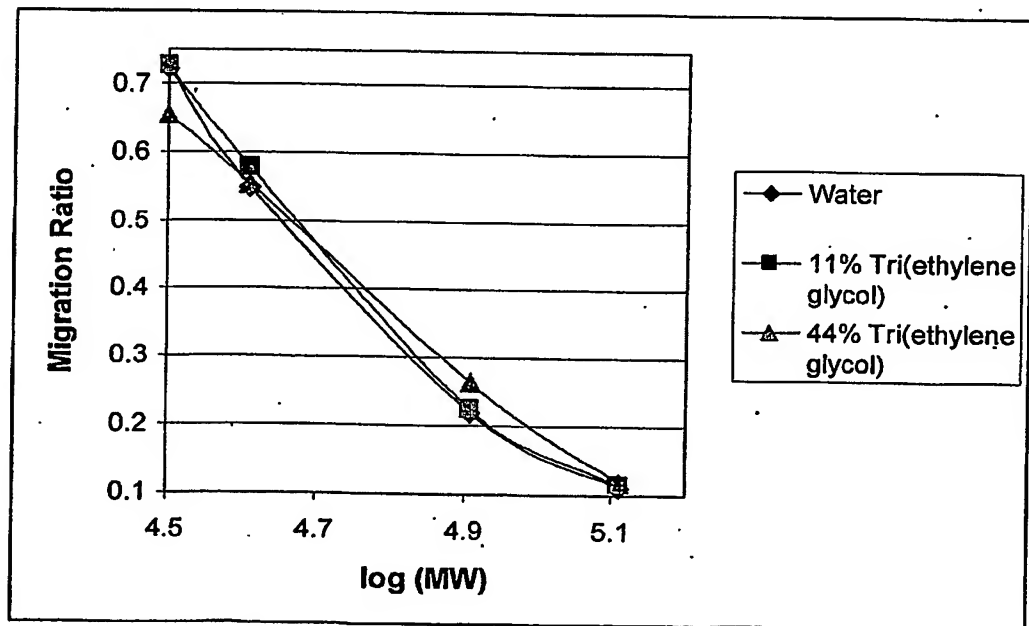


Figure 4

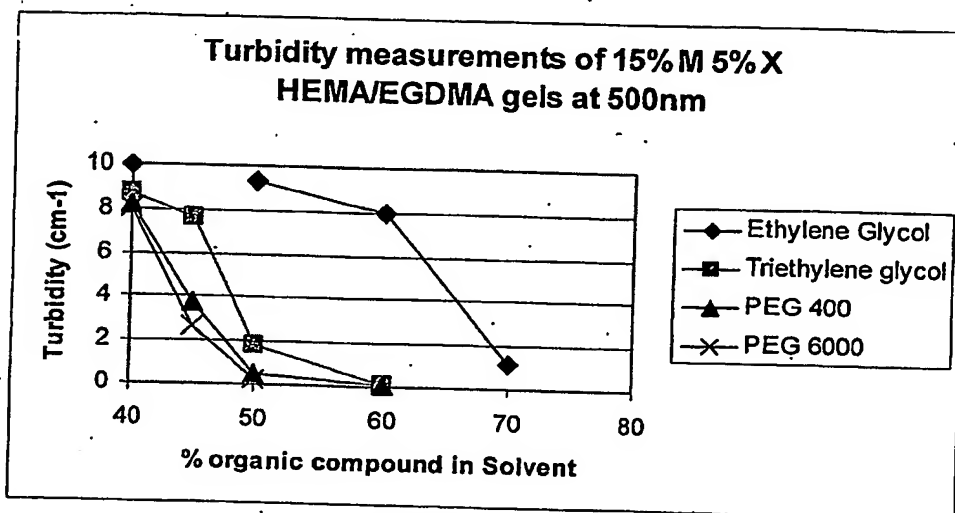


Figure 5

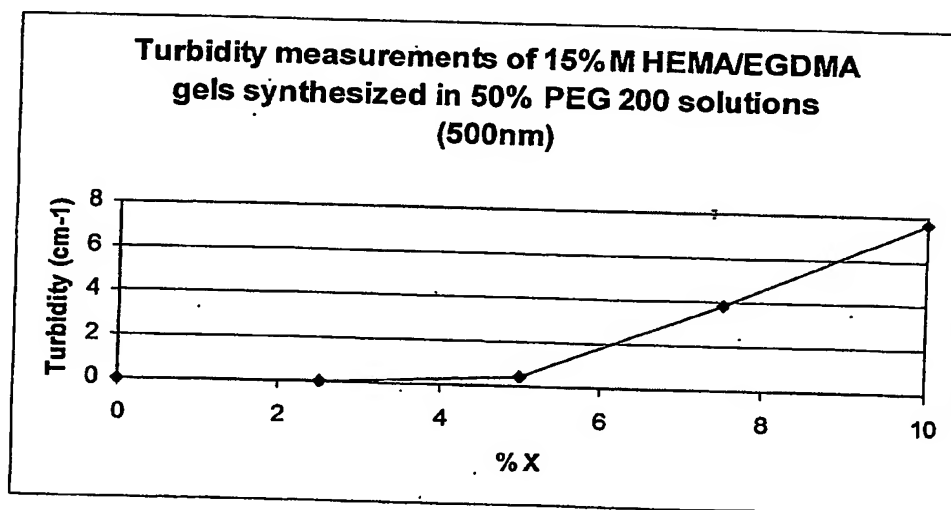


Figure 6

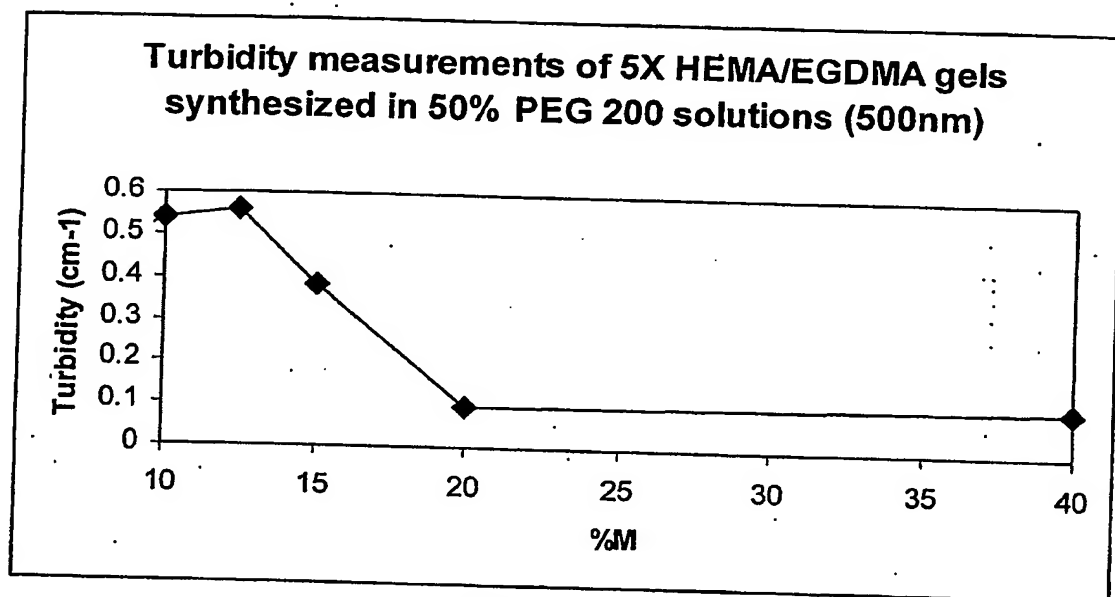


Figure 7

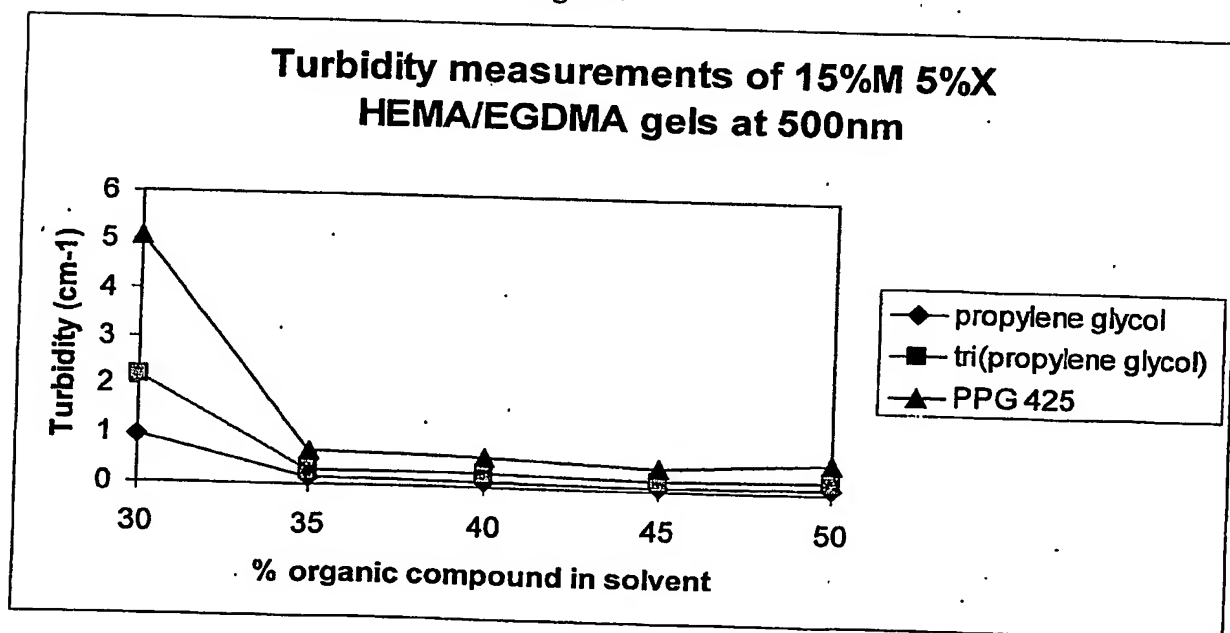


Figure 8

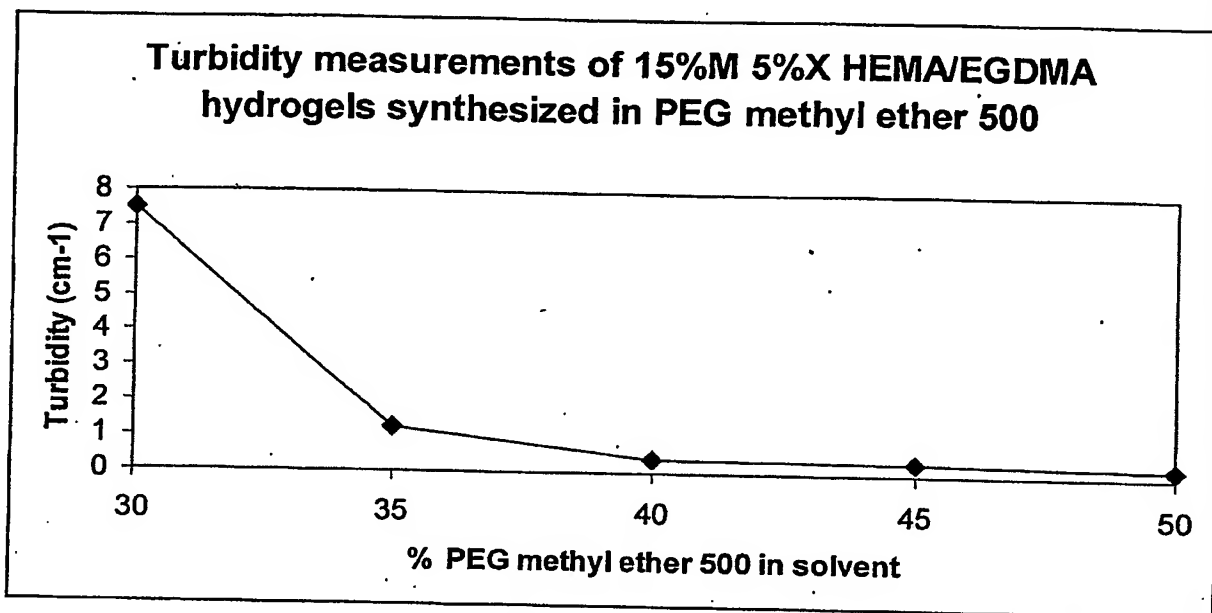


Figure 9

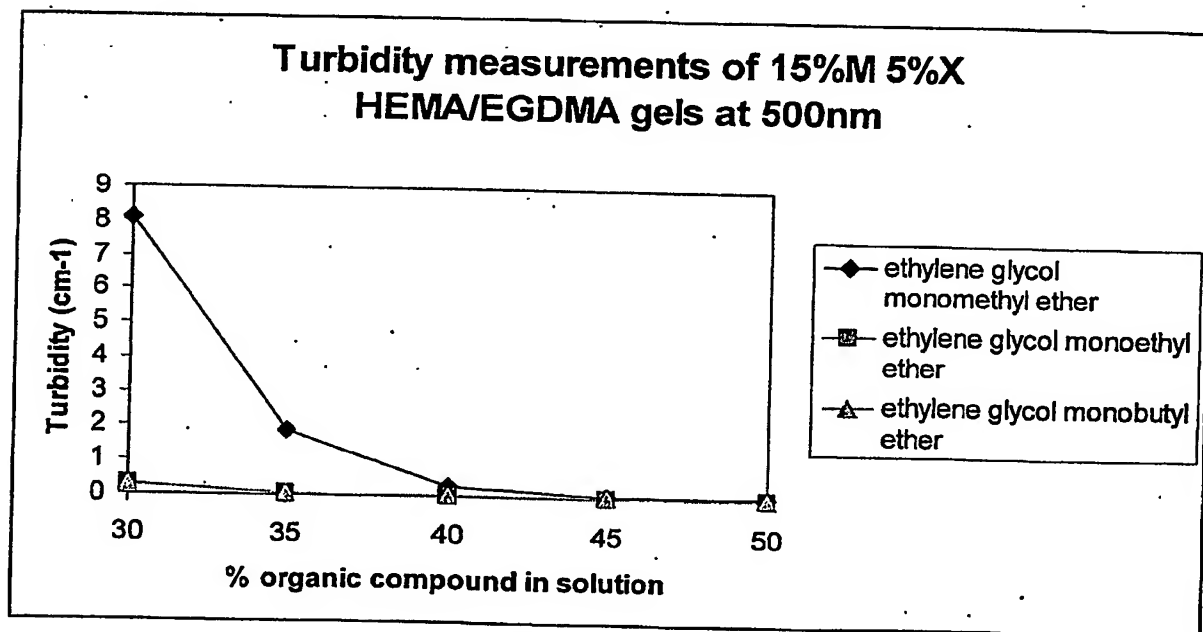


Figure 10

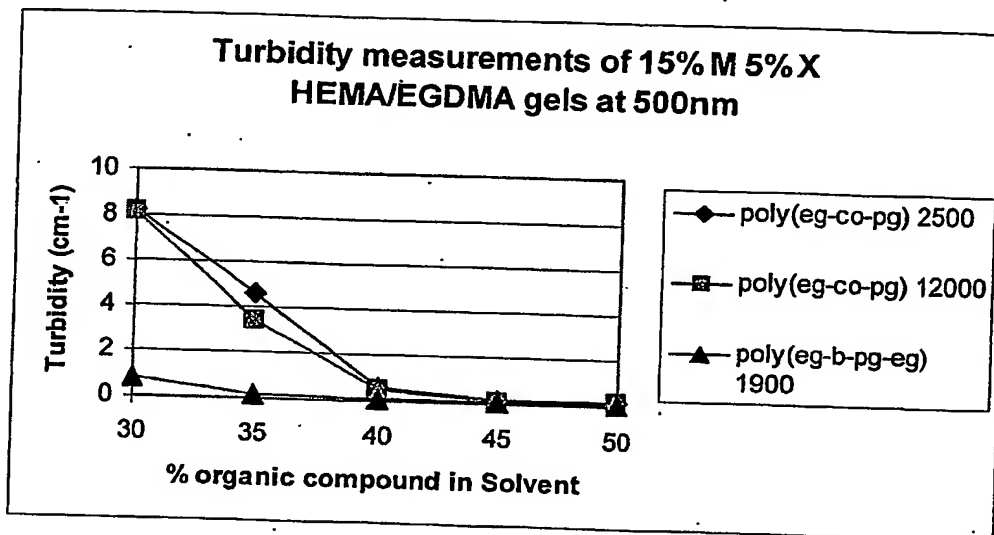


Figure 11

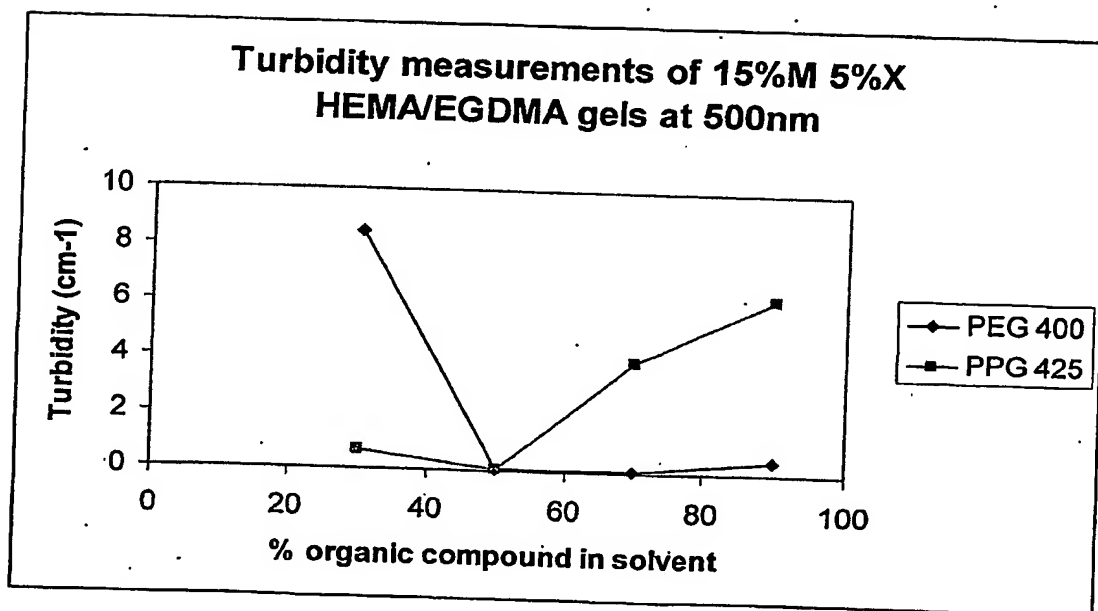


Figure 12

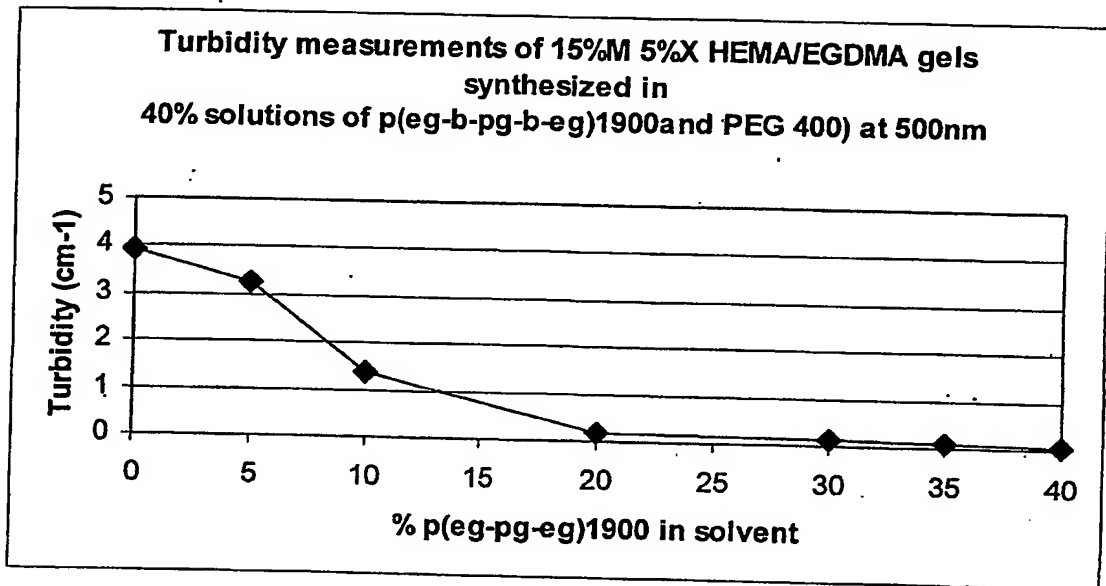


Figure 13

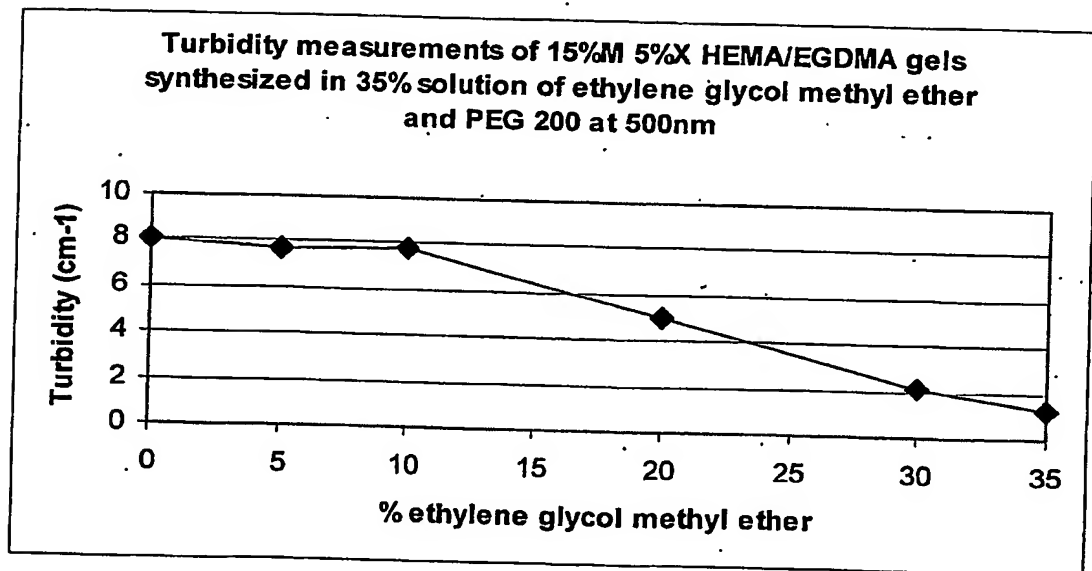


Figure 14

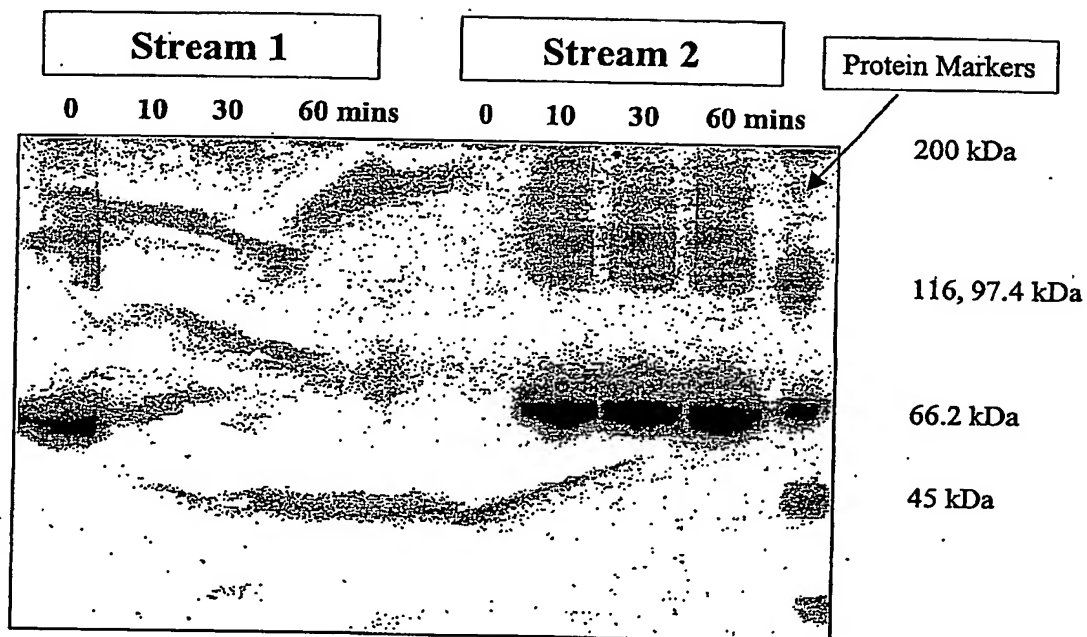


Figure 15

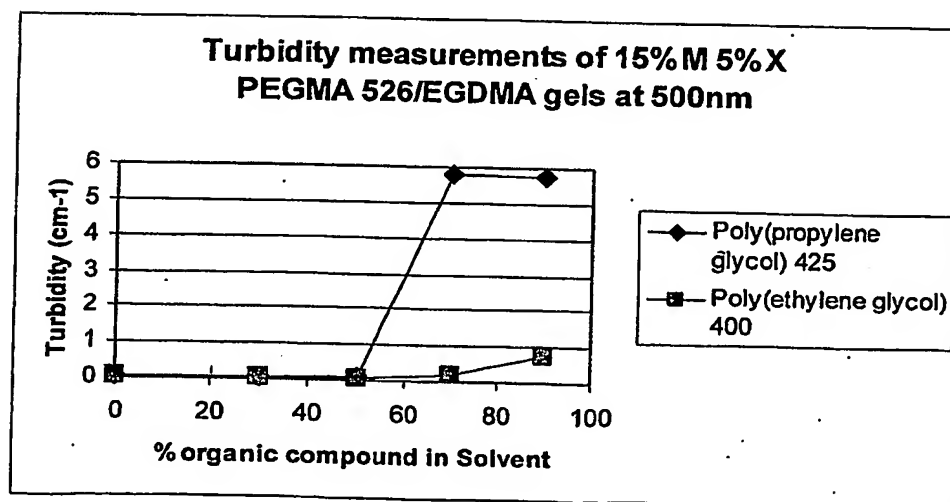


Figure 16

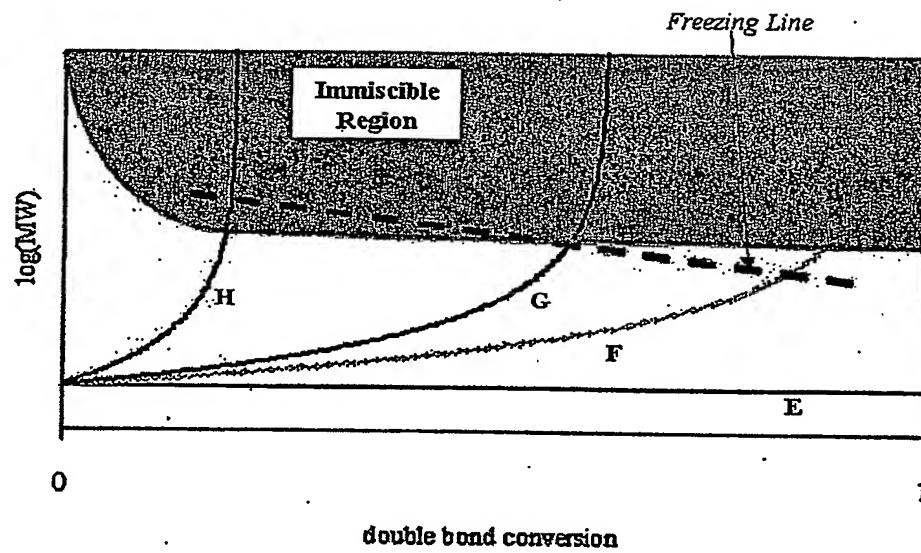


Figure 17

10/10

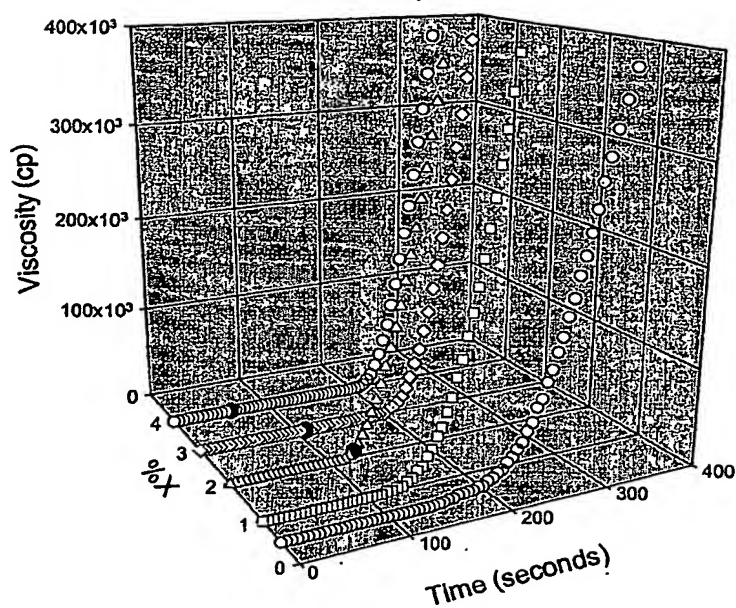


Figure 18

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